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TOLERANCE INDUCTION AND TRANSPLANTATION OF THE MOUSE HEART

by

© DENNIS WILLIAM JIRSCH

A THESIS

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IN

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled TOLERANCE INDUCTION AND TRANSPLANTATION OF THE MOUSE HEART submitted by DENNIS WILLIAM JIRSCH in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in EXPERIMENTAL SURGERY.

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Date September 28, 1973

This text is dedicated to two extraordinary, gentle men:

Fred Proudfoot, and a man called Gumbo.

ABSTRACT

Specific and consistent means of achieving long-term or permanent survival of allografted organs remains an elusive goal. The experimental induction of allograft tolerance has been studied most extensively in mice with skin grafts, in rats with renal allografts and in larger outbred species with a variety of organs. Experiments in outbred species are often difficult to interpret and the advantages of the defined histocompatibility systems in the mouse and rat are evident. Rat renal allografts are, however, technically difficult and time consuming, and the inordinate ease with which kidney survival may be obtained across the Ag-B locus, make general conclusions suspect. Skin allografts are, on the other hand, technically simple but are difficult to evaluate functionally. They contain numerous cell types, including lymphocytes, and, exposed as they are to the microbial environment, may become infected and valueless in the presence of irradiation or other non-specific forms of immunosuppression.

For these reasons transplantation immunity and tolerance have been studied in the mouse, using fetal cardiac allografts. Fetal hearts removed from Balb/c mothers after 16-18 days gestation were transplanted into the subcutaneous ear tissue of syngeneic or allogeneic (CBA) adult mice. Evaluation by electrocardiography and visual observation of pulsatile activity permitted precise functional assessment of graft survival. While syngeneic grafts survived indefinitely,

allogeneic grafts (Balb/c-CBA) were rejected within seven days and repeat grafts elicited an anamnestic response. Histologic evidence of graft rejection occurred later than functional death, as did peak levels of humoral cytotoxic antibody. Spleen cells removed from heart allografted animals at varying periods after transplantation were tested *in vitro* for their ability to effect cell mediated killer activity. Single heart allografts did not engender sufficient host cellular immunity to produce significant release of ^{51}Cr from radiolabelled Balb/c tumor cells *in vitro*.

Permanent survival of Balb/c cardiac allografts in adult CBA hosts was produced in several ways. It was found that lethally irradiated (950R), heart allografted mice reconstituted with syngeneic bone marrow would reject an allograft heart within four to five weeks. Reconstitution was subsequently performed with stem cell rich fractions of spleen and bone marrow, derived by velocity sedimentation cell separation techniques. Reconstitution of irradiated allografted animals with stem cell rich fractions of spleen, but not bone marrow, produced small but consistent numbers of animals bearing functional allografts beyond 12 weeks. Further, if mice reconstituted with syngeneic marrow were treated within the first few weeks post-irradiation with heterologous or homologous antisera or the gamma globulin fraction thereof, immunosuppression was obtained. Depending on the treatment, variable numbers of animals retained functional allografts 12 weeks post-grafting. Such animals, not subject to continuing immunosuppression, did not subsequently reject their allo-

grafts and were considered tolerant.*

Spleen cells from tolerant animals were fully immunocompetent and, *in vitro*, could be sensitized in the presence of graft alloantigens (Balb/c tumor cells) to effect subsequent killer activity. This suggested that a serum factor(s) or cell prevented immune expression of potential antigen recognition *in vivo*. Sera from tolerant animals were, accordingly, added to allograft type (Balb/c) fibroblast monolayers subjected to CBA-spleen killer cell attack. Tolerant serum did not prevent the immune destruction of fibroblasts by sensitized cells. Small numbers of lymph node cells ($2\text{-}10\times10^6$) removed from tolerant animals did, however, consistently transfer tolerance when injected into syngeneic irradiated allografted mice reconstituted with whole bone marrow.

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*Allograft tolerance in this thesis therefore considered as an operational term, not necessarily descriptive of a unique mechanism for allograft survival.

PREFACE

The subject of this thesis relates to a matter of increasing biolgical and clinical interest, that of producing specific host unresponsiveness or tolerance to a foreign graft. The very large body of knowledge pertinent to this topic is difficult, indeed, to summarize with satisfaction. The first chapter of this text attempts, nevertheless, to document certain relevant concepts and experimental data in this regard. The second chapter provides a detailed description of the materials and methods encountered throughout the text so that, in the interests of the reader, subsequent brief mention will suffice. The remaining chapters constitute the "benchwork" of the thesis; each has been constructed individually as a study in depth of one aspect of the major work. The concluding chapter details summarily the perceptions of this research, proposes further avenues of investigation and attempts to reconcile this particular endeavor within the context of major inquiry into the development of transplantation tolerance. The appendix contains data which have emerged, in branch fashion, from the trunk of the thesis effort.

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CHAPTER I

STUDIES IN TRANSPLANTATION TOLERANCE - AN OVERVIEW

A. Introduction

Clinical organ transplantation has achieved remarkable success in recent years. Transfer of a kidney from either a living related donor or a cadaver to a patient with end stage renal disease has now become an accepted therapeutic measure. As the technical problems in transplantation surgery become ever less burdensome, it is clear that the immune response of a patient to a foreign graft persists as the major obstacle preventing widespread allotransplantation therapy of a variety of diseases. The immunologic factors important in allograft acceptance appear to be threefold and include proper donor selection, effective immunosuppression and the induction of immunological tolerance. The benefit of histocompatibility between donor and recipient is indicated by several analyses of the results of tissue typing in renal allograft patients (Patel, Mickey *et al*, 1968; Festenstein, Sachs *et al*, 1971). As more antigenic groups and immune response genetic loci are identified, however, the probability of finding fully compatible donor recipient pairs will diminish. In the unrelated cadaver donor situation a residue of histoincompatibility must be neutralized, either by immunosuppression or the induction of tolerance. The immunosuppressive agents now in use, however, are non-selective and seriously impair host resistance to the microbial environment, suppress vigilant

immunologic surveillance mechanisms and are associated with unique toxicity problems.

The induction of specific graft directed tolerance is thus the ultimate goal of the transplantation biologist. This chapter will briefly survey significant work in this regard, describe in some detail laboratory systems which permit the induction of specific unresponsiveness both *in vivo* and *in vitro*, and conclude with speculations which determined the subsequent research described in this text.

B. Antigen Recognition and the Origin of Immunocompetent Cells

1. The Lymphocyte, the Spearhead of Immune Defence

The lymphoid system constitutes a specific adaptive mechanism whereby confrontation with an extrinsic antigenic molecule provokes lymphocytes to discriminate between "self" and "foreign" and leads to the production of antibody forming cells and/or specific "killer" cells capable of destroying foreign tissue on direct contact. Only a small proportion of lymphoid cells are genetically committed to react against a given antigenic specificity (Burnet, 1959), and Medawar (1960) has defined an immunocompetent cell as one capable of recognizing antigen. Once activated by antigen, these cells enlarge, divide and differentiate into a population of pyronin positive cells which eventually give rise to further small lymphocytes (Gowans, McGregor *et al*, 1962). In the case of foreign graft tissue, this progeny of specifically sensitized lymphocytes is concerned with the actual destruction of the transplant (cell-mediated immunity). Other responses are expressed through

circulating antibodies derived from descendents of another class of lymphocytes which have, through differentiation and multiplication, developed the intracellular machinery necessary for antibody production (humoral immunity). The end point of this process is the antibody secreting plasma cell.

Certain lymphocytes develop within the thymus and are termed T cells, while others differentiate within bone marrow, independent of thymic influence, and are designated as B cells. For most, but not all antigens the cooperation of these two cell types is necessary for an antibody response (Miller and Mitchell, 1969; Davies, 1969; Claman and Chaperon, 1969; Taylor, 1969). T cells evidently serve in a helper capacity in presenting antigen to relevant B cells, which are the immediate precursors of antibody forming cells (Nossal, Cunningham *et al*, 1968). Other T cells become specifically activated upon contact with antigen and are the effector cells in cell-mediated immunity (Cerottini, Nordin *et al*, 1970; Miller, Brunner *et al*, 1971). Neonatal thymectomy thus greatly depresses both cell-mediated immunity and humoral immunity to those antigens which require T-B cell cooperation (Miller, 1961; Miller and Osoba, 1967). Certain antigens do not require T cell help and humoral immunity in these instances remains intact following thymectomy (Miller and Osoba, 1967). These considerations are schematically represented in Figure 1.

To initiate the events leading to either humoral antibody production or specifically sensitized "killer" cells, immunocompetent cells must be triggered by foreign molecular structures, either soluble antigens or antigenic determinants on cell surfaces. What are the

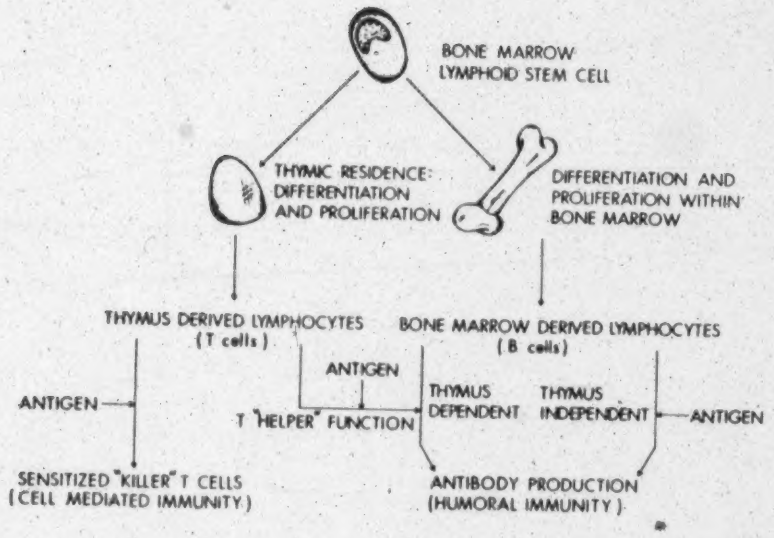


Figure 1 Development of specialized T (thymus derived) and B (bone marrow derived) lymphocytes and their relationship to cellular and humoral immunity.

entities on lymphoid cells capable of discriminating between "self" and "nonself" and how does this recognition operate? The lymphocyte surface can be regarded as a switchboard from which signals are transmitted into the cell interior. Signals originate from molecular structures termed antigen recognition sites, which are sterically complementary to the structure of antigen molecules. In the case of B cells, these recognition sites are of monomeric immunoglobulin IgM in character and are probably identical in basic structure with antibody. Helper T cells also appear to have specific surface receptors for antigen but T cell surface antibody is not readily demonstrable and differs from that found on B cells both qualitatively and quantitatively (Greaves and Hogg, 1971). There is evidence, however, that immunoglobulin light chains form a part of the T cell receptor for antigen. Antilight chain sera pretreatment of T cells can inhibit the specific "irradiation suicide" of such cells subsequently incubated with ^{125}I labelled antigen (Basten, Miller *et al*, 1971). Cooper and Ada (1972) have also reported that antilight chain sera could inhibit the suicide of T cells involved in delayed type hypersensitivity reactions.

2. Origin of the Immunocompetent Cell

What is the origin of lymphoid cells capable of recognizing a particular antigen and how is this potential generated? All cells of the hematopoietic and lymphoid systems are derived from self perpetuating bone marrow stem cells. Grafting of bone marrow into an animal which has received lethal x-irradiation (with consequent destruction of its own hematopoietic tissue) is a life saving

procedure. Indeed, repopulation studies of Ford (1966), Micklem, Ford *et al*, (1966) and Globerson and Auerbach (1967) showed that bone marrow has the capacity to recolonize not only bone marrow, but also the thymus and peripheral lymphoid tissues. Further studies by Wu, Till *et al* (1968) demonstrated that single stem cells were capable of differentiation along lymphoid, erythroid, granulocytic and megakaryocytic lines.

It is evident that somewhere along the developmental pathway, from a few precursor cells (Figure 2) to the immensely large pool of specific immunocompetent cells in the lymphoid system, there occurs a critical phase during which the lymphocyte acquires the capacity to recognize a particular antigen and becomes immunocompetent. Since there are, at the very least, several thousand antigenic structures and each lymphocyte recognizes only one antigenic specificity, we are faced with the problem that a single lymphocyte precursor must bear genetic information for the synthesis of several thousand different recognition sites. The hypothesis of Burnet (1959) has provided an answer to the dilemma. A limited number of genes undergo random somatic mutation during lymphopoiesis. The large number of mutant lymphocytes produced in this way are each genetically capable of recognizing a specific antigen. The potential for synthesis of recognition sites complementary to a given antigen then becomes the property of a single clone of cells. (This theory is known as the "clonal selection theory of immunity".) A single lymphoid precursor cell should thus generate a population of cells with gradually diversifying antigen recognition specificities. Yung, Wyn-Evans *et al*

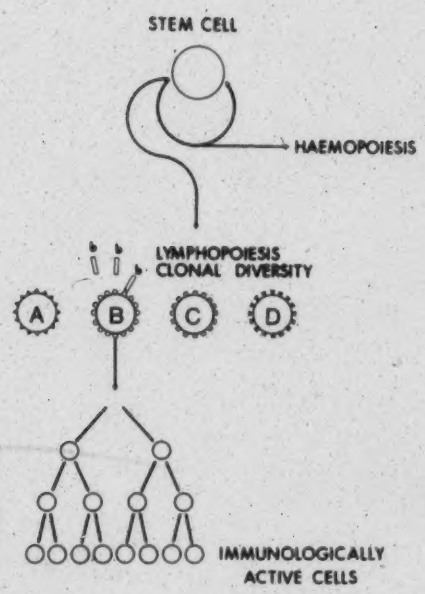


Figure 2 The origin of immunocompetent cells. A, B, C and D are immunocompetent cells belonging to different cell clones. Each clone possesses recognition sites specific for only one antigen. b= specific antigen interacting with an immunocompetent cell.

(1973) have, in fact, monitored the ontogenic development of immunocompetent cells in the mouse, and have found that a full antigen recognition spectrum is expressed within 20 days of clonal expansion from a single embryonic stem cell.

C. Specific Suppression of the Immune Response

1. Immunological Tolerance

a. Self tolerance

Immunological tolerance has been defined by Dresser and Mitchison (1968) as a state of partial or complete incapacity to respond to an immunogenic stimulus brought about by prior contact with antigen. The best example of this immunological unresponsiveness is the tolerant state we maintain toward our own body constituents. Attention was first directed to this phenomenon by Owen in 1945, who observed that dizygotic cattle twins are generally chimeric with respect to their red blood cell types due to sychorial placental anastomosis, which would permit free exchange of fetal blood *in utero*. Burnet and Fenner (1949) postulated shortly thereafter that a function of the lymphoreticuloendothelial system during embryonic life was to distinguish self from nonself constituents. Consequently, the presentation of foreign material to the developing embryo before it has learned to make this distinction would engender a specific lack of reactivity to this antigen. Experiments of Billingham, Brent *et al* (1954), confirmed this interpretation. Skin grafts were permanently accepted between different inbred strains of mice if graft recipients were inoculated *in utero* with living donor spleen or kidney cells. The

period during which tolerance may be induced during ontogeny of the immune system varies according to species, the tolerance responsive period extending several weeks after birth in rats (Woodruff and Simpson, 1955), while sheep embryos become immunocompetent well before parturition (Schinkell and Ferguson, 1953).

The role of the thymus in the induction and maintenance of the tolerant state has not yet been unequivocally established, but it is clearly important. Burnet (1962) suggested that one of the thymic functions was either the elimination or inactivation of self reactive clones. Antigens presented to the thymus would, therefore, be recognized as "self" and not subject to immune attack. In accord with this concept, a number of investigators (Staples, Gery *et al*, 1966; Isakovic, Smith *et al*, 1965; Taylor, 1969) have been able to produce tolerance by direct intrathymic injection of antigens. The importance of this data is unclear since antigen specific tolerance can be induced in adult thymectomized animals (Mitchison, 1967) and the thymus does contain small numbers of lymphocytes capable of initiating an immune response (Mitchell and Miller, 1968). The suggestion is, therefore, that mature immunocompetent lymphocytes represent target cells in tolerance induction and that the thymus is not the only site available for this interaction.

2. Methods of Inducing Tolerance *In Vivo*

In experiments involving extrinsic antigens, several factors have been found important for tolerance induction. The route of antigen administration is important, for example, and tolerance is induced more easily following intravenous administration of the antigen rather than

subcutaneous, intradermal or intraperitoneal injection. The experiments of Triplett (1962) have suggested, moreover, that the permanent presence of antigen is necessary for the maintenance of tolerance. This is true probably because immunocompetent cells are generated throughout life and may replace tolerant cells unless they are rendered unresponsive themselves.

Ever since the experiments of Smith and Bridges (1958), two factors have been found critically important in tolerance induction: concentration and the molecular structure of the antigen used. For many antigens, there exist two distinct zones of concentration which can induce tolerance, a high and a low dosage zone, while intermediate concentrations provoke an immune response. This phenomenon has been characterized in experiments done with bovine serum albumin in adult mice and rabbits (Mitchison, 1964; Thorbecke and Benacerraf, 1967) and with the *Salmonella* flagellar antigens in rats (Shellam and Nossal, 1968; Ada and Parish, 1968). Immunological tolerance corresponding to these two zones has been defined as high zone and low zone tolerance.

The molecular state of most antigens is of paramount importance in determining the capacity to induce tolerance. Dresser (1962), for example, showed that monomeric (deaggregated) human gamma globulin, when injected into certain strains of mice rendered them unresponsive to subsequent injections of the aggregated human gamma globulin, a potent immunogen. Parish, Wistar *et al* (1969) have reported the isolation of fragments of cyanogen bromide treated flagellin (mol wt 40,000) of *Salmonella adelaide*. One of the fragments (Fragment "A", mol wt 18,000) retains the main antigenic activity of flagellin and

induces tolerance but not immunity to flagellin when injected repeatedly at a particular dose level. Antigens may, therefore, exhibit tolerogenic or immunogenic qualities when administered *in vivo*. Since tolerogens can be converted into immunogens by nonspecific means such as by mixing them with adjuvants, Dresser (1963) has suggested that immunogenic antigens have properties called "adjuvanticity", which, in the case of the bovine gamma globulin system, could be separated as aggregated material.

Tolerance to normally immunogenic substances can be induced in adult animals by administration of the antigen along with x-irradiation, immunosuppressive treatment (including antilymphocyte globulin) and by mechanical lymphocyte depletion (thoracic duct cannulation). Tolerance induction and immunity, though alternative effects at the single cell level, often coincide in adult animals. A balance between the two phenomena can apparently swing toward tolerance induction by nonspecific removal of immunocompetent cells. This situation is most akin to that of the developing fetus: apparently a stage of immunocompetence most susceptible for tolerance induction during which stem cells proliferate and differentiate into mature lymphoid elements. Mitchison (1967) has shown that the threshold of antigen concentration for induction of tolerance under such conditions was 10^{-8}M for several different antigens. It has been calculated, moreover, that the dose of antigen per kilogram of body weight necessary to induce tolerance in an adult irradiated animal is comparable to that required in the neonate (Smith, 1961).

As for potential clinical use, antilymphocyte serum as a means of immunosuppression during tolerance induction has been favored over x-irradiation for obvious reasons. For instance, rabbit anti-mouse

antilymphocyte serum produces prolonged and profound lymphopenia and grossly deficient immune reactivity when injected into mice. Animals treated in this manner can be rendered tolerant to transplantation antigens of another mouse strain by injecting them with allogeneic spleen cells, similar to the phenomenon described in the neonate (Billingham and Brent, 1957). Recipients will then specifically retain skin grafts of the donor spleen cell strain. Since, as discussed earlier, thymectomy results in immunologic deficiency, tolerance is most easily induced by treatment of adult thymectomized mice with antilymphocyte serum prior to the administration of the tolerance inducing spleen cells (Monaco, Wood *et al*, 1965). Adult thymectomized ALS-treated mice rendered tolerant by large doses of allogeneic lymphoid cells exhibit stable lymphoid chimerism (Monaco, Wood *et al*, 1966) and a long-standing tolerance. In contrast, non-thymectomized mice rendered tolerant by ALS treatment accompanied with donor marrow infusion do not demonstrate long-standing lymphoid cell chimerism and the tolerant state is of short duration (Wood, Gozzo *et al*, 1972).

a. Are both T and B cells involved in tolerance?

From the studies of Chiller, Habicht *et al* (1970), it appears that both B and T cells can be rendered tolerant. Neither thymus nor bone marrow cells from A/J mice injected three weeks previously with tolerogenic human gamma globulin could, with their normal counterpart, reconstitute secondary irradiated syngeneic recipients. With extrinsic proteins, both Mitchison (1971) and Rajewsky (1971) have found that the response of mice which exhibited low zone tolerance could be restored with the transfer of activated T cells but that high zone

induced tolerance was unaffected. High zone tolerance may thus be due to specific inactivation of both T and B cells with low zone tolerance induction dependent only on T cell inactivation. In agreement with this, Chiller, Habicht *et al* (1971) have found that T cells could be rendered tolerant *in vivo* to human gamma globulin with concentrations of antigen far too low to specifically inactivate B cells.

Studies on tolerance induction *in vivo* have not answered the fundamental question as to the fate of the tolerant cell. Irreversible inactivation or cell death has been regarded as most consistent with current immunologic data but there is recent alternative evidence which, in fact, considers at least certain types of immunological tolerance as an active state of cell repression rather than elimination (McCullagh, 1970a,b; 1972). Normal syngeneic lymphocytes could not restore immune reactivity when transferred to hosts tolerant to sheep erythrocytes and were, indeed, rendered unresponsive themselves. Allogeneic lymphocytes exposed to tolerant lymphocytes could, however, restore the immune response suggesting an "unmasking" of immunocompetent cells. In accord with this concept Gershon, Cohen *et al* (1972) have found T cells capable of specific immunosuppression. Nisbet (1971) has produced tolerance by parabiotic union of parental and F_1 mice and has been able to selectively transfer tolerance by injecting large numbers of tolerant thoracic duct lymphocytes or spleen cells into neonatal recipients, subsequently given a skin graft.

3. Antibody Mediated Enhancement and the Immune Response

The presence of humoral antibodies directed toward graft antigens can sometimes facilitate the growth of foreign tumor cells which

would normally be rejected (Kaliss, 1956; 1958). Enhancing antibodies can be raised in response to normal immunization procedures (active enhancement) or the sera of animals previously exposed to tumor antigens may be passively administered to second graft recipients (passive enhancement). Kaliss (1956), for example, showed that serum from tumor bearing mice would promote tumor growth in secondary hosts if administered up to one week before or after the tumor inoculum. Although initial work on enhancement involved allografted tumors in rodents, the phenomenon has become relevant as a means of specifically facilitating the survival of allografted organs. Three main possibilities have been proposed as mechanisms by which enhancement may work:

a. Afferent blockage

Graft directed antibodies may cover antigenic determinants on foreign cells, thereby preventing activation of relevant immunocompetent cells (Uhr and Möller, 1968). For example, Snell, Winn *et al* (1960) studied the lymph nodes draining the site of an allogeneic tumor. If mice were injected with hyperimmune antitumor antiserum before the tumor was transplanted, the lymph nodes were less reactive to the tumor thereafter, suggesting that the antiserum had, indeed, covered tumor cell antigens. On the other hand, several workers (Terres and Wolins, 1961; Segre and Kaeberle, 1962) have demonstrated that specific antibody administered together with antigen may increase rather than diminish antigen immunogenicity. Similarly, Diener and Feldmann (1970) showed that polymerized flagellin from the bacterium *Salmonella adelaide* in the presence of antibody excess displayed an

in vitro immune response comparable to controls stimulated with antigen alone. Uhr and Baumann (1961) in work with the tetanus toxoid antigen, could inhibit an antitetanus response with antibody, but with a quantity of antitoxin sufficient to cover only a small fraction of antigenic sites on the administered toxoid. Haughton and Nash (1969) provided further evidence against peripheral enhancement. The number of antibody molecules necessary to cover the antigenic sites of 5×10^8 sheep erythrocytes was 100 fold greater than that needed to suppress the immune response. Tumor allografts, moreover, can be enhanced by administration of antiserum at the time of peak rejection response (Kaliss, 1958) and, as shown by Möller (1965), smaller doses of antiserum were generally more effective than large doses in promoting tumor growth. Enhancing antiserum, moreover, suppressed the lymphocytosis accompanying allograft tumor immunity, in cases where surgical excision of tumor was ineffective (Takasugi and Hildemann, 1969).

b. Central immunosuppression

Humoral antibodies may act directly on immunocompetent cells to specifically decrease immunological reactivity. This has been demonstrated convincingly by Amos, Cohen *et al* (1970) using a transplantable mouse tumor. Tumor cells were incubated with antiserum, washed free of excess serum, and then mixed with lymphoid cells from mice sensitized against the tumor. When these lymphoid cells were added to fresh tumor cells and injected into recipient mice, tumor growth was enhanced. Control experiments with lymphoid cells exposed

to tumor and nonimmune serum, failed to facilitate tumor growth. The work of Rowley, Fitch *et al* (1969a,b) is confirmatory and suggests that antibody may directly reduce the number of immunocompetent cells initially responsive to antigen.

c. Efferent enhancement

Humoral antibodies bound to the surface antigens of foreign cells considered as targets, conceivably protect such cells from the killing activity of sensitized lymphocytes. The first clear demonstration of efferent enhancement was given by Möller (1963) who grafted two identical homologous tumors simultaneously into the same nonsensitized mouse. One graft had been exposed *in vitro* to specific antiserum and this showed enhanced growth; the second, untreated graft was rejected in normal fashion. Further support for efferent blockage has been given by the recent work of the Hellströms. In tumor systems, sera of animals (Hellström and Hellström, 1969; Hellström, Evans *et al*, 1969; Hellström, Hellström *et al*, 1970a) or patients (Hellström, Hellström *et al*, 1970b) bearing antigenically distinct tumors were shown to contain a factor that specifically inhibited the *in vivo* antitumor activity of lymphocytes from the tumor bearer or lymphocytes from specifically sensitized animals. Similarly, the sera from female mice mated with allogeneic males inhibited the *in vitro* activity of lymphocytes sensitized to paternal strain tissue (Hellström, Hellström *et al*, 1969). A serum factor that abrogated the activity of sensitized lymphocytes has been demonstrated in chimeric dogs and mice (Hellström, Hellström *et al*, 1970c; Hellström, Hellström *et al*, 1971) and renal

allografted humans (Quadracci, Hellström *et al*, 1971; Hellström and Hellström, 1972a).

Although the initial proposal (Hellström and Hellström, 1970) was that the serum factor inhibiting the *in vitro* activity of sensitized lymphocytes was an antibody which combined with antigens on the target cell surface (efferent enhancement), a central form of immunosuppression was later postulated (Hellström and Hellström, 1971) for the so-called "blocking antibody". Ingenious experiments of Sjögren, Hellström *et al* (1971) indicated that blocking antibody might, in fact, be a complex of antigen and antibody.

Enhancing sera from mouse sarcomas were absorbed by tumor cells, which were spun down and resuspended in buffer. After mixing in buffer for one hour, tumor cells were discarded and the supernatant was passed through an ultrafilter which would retain molecules of molecular weight greater than 100,000. The filtrate was then passed through a second filter capable of retaining molecules of molecular weight greater than 10,000. Only a 1:1 combination of the material retained by the two filters optimally prevented lymphocyte mediated lysis of target cells, and neither of the two fractions alone were fully immunosuppressive. It was suggested that the two components separated by the molecular filters may have represented antigen and antibody and that uniting these two had led to blocking antigen-antibody complexes. This interpretation is supported by other experiments, in particular the work of Stuart and colleagues (Stuart, Saitoh *et al*, 1968; Stuart, Bastien *et al*, 1970). Rat F_1 hybrid kidneys were grafted to parental strain animals. Graft recipients were inoculated with spleen

cells of graft donor type in combination with homologous antiserum directed against graft alloantigens. Maximal allograft enhancement was achieved only when antiserum treatment was combined with the administration of donor spleen cells and, with this regimen, permanent survival of the transplanted kidneys was obtained in many recipients.

French and Batchelor (1969) transplanted allogeneic (F_1 to parent) rat kidneys in similar manner, but could enhance graft survival with passive administration of graft directed antiserum alone. It is possible however, that graft derived antigen itself may have permitted antigen-antibody complex formation. Enhanced graft survival has been obtained, moreover, in transplantation of skin, kidneys or cardiac allografts in several laboratory species by injection of donor blood or blood elements at different intervals before and after grafting (Halasz, 1963; Marino and Benaim, 1958; Halasz, Orloff *et al*, 1964; Marquet, Heystek *et al*, 1971). Since the antibody response to rat renal allografts is not abrogated by passive administration of graft directed antisera (Lucas, Markley *et al*, 1970), donor blood may indeed provide a ready source of antigen for complex formation with antibody. In the tumor cell system of Amos, Cohen *et al* (1970), described above, a similar combination of tumor cell antigen and specific antibody exposed to lymphocytes suppressed their ability to kill new cells. The combination of tumor plus antibody was more effective than exposure to either tumor or antibody alone. These studies were facilitated by a tissue culture system which allowed the maintenance of dispersed mouse lymphoid cells *in vitro*.

The concept of serum mediated blocking factors preventing the

expression of cell mediated immunity is now firmly established in tumor and graft systems. The relationship of this phenomenon to the induction of neonatal tolerance or to normal self tolerance is not clear. There is, however, recent evidence that mice may react against antigens present on their normal (brain) cells *in vitro*, and expression of such induced cell mediated immunity is prevented by blocking factors present in normal sera (Hellström and Hellström, 1972b). This evidently contradicts the classic explanation for both neonatally induced tolerance and normal tolerance to self antigens, whereby lymphoid clones genetically capable of immune reactivity are eliminated during embryonic development.

A new concept in specific immunosuppression emanates from the work of Ramseier and Lindenmann (1972). Their work in the field of acquired tolerance may be relevant to self tolerance between lymphocytes. Antibodies can be raised against specific antigen receptors present on lymphoid cells, in their notation termed anti-RS, where RS signifies recognition structure. The specificity of these antibodies has been demonstrated in the following way. F_1 hybrid mice were immunized with repeated small doses of parental lymphoid cells, testing the subsequent serum for antibodies to histocompatibility antigens. They reasoned that F_1 cells would not bear receptors for either parental cell types but that parental cells themselves would bear receptors for the other parent. This parental recognition site directed toward the other parental lymphocyte would be the only unique structure that F_1 progeny could recognize and produce antibody against. The presence of these anti-RS antibodies has been demonstrated in several situations. The

most convincing finding was the ability of anti-RS sera to inhibit graft-versus-host reactions (Joller, 1972). These anti-RS sera did not, however, inhibit mixed lymphocyte culture reactions. The relation of anti-RS sera to blocking factor is not yet clear but may conceivably be important, especially in transplantation of lymphoid tissues.

4. *In Vitro* Manipulation of the Immune Response

a. Tolerance induction *in vitro*

The abundant *in vivo* data relative to immunoregulative mechanisms have led to a number of interpretations which are conceptually disturbing. This has prompted experimentation by Diener and colleagues in an attempt to clarify the role, at the cellular level, of free antibody and of antigen-antibody complexes in immune suppression. Experiments were done on dispersed lymphocytes from mouse spleen which were kept alive using a tissue culture system devised by Marbrook (1967) (Figure 3). Spleen cells in an adequate medium were placed in a cylindrical tube closed off at the bottom by a dialysis membrane. This tube was then placed in an Erlenmeyer-type flask containing sufficient tissue culture medium to equal the fluid level of the cell suspension in the inner cylinder. Cultures were maintained in a humidified incubator at 37°C with a constant flow of a gas mixture consisting of CO_2, O_2 and N_2 (Mishell and Dutton, 1967). Direct interaction of splenic lymphocytes with antigen and/or antibody was thus permitted. Use was made of the H (flagellar) antigens of *Salmonella adelaide* (Ada, Nossal *et al*, 1964) which may be obtained in three different forms: polymerized flagellin (POL; mol wt nx40,000),

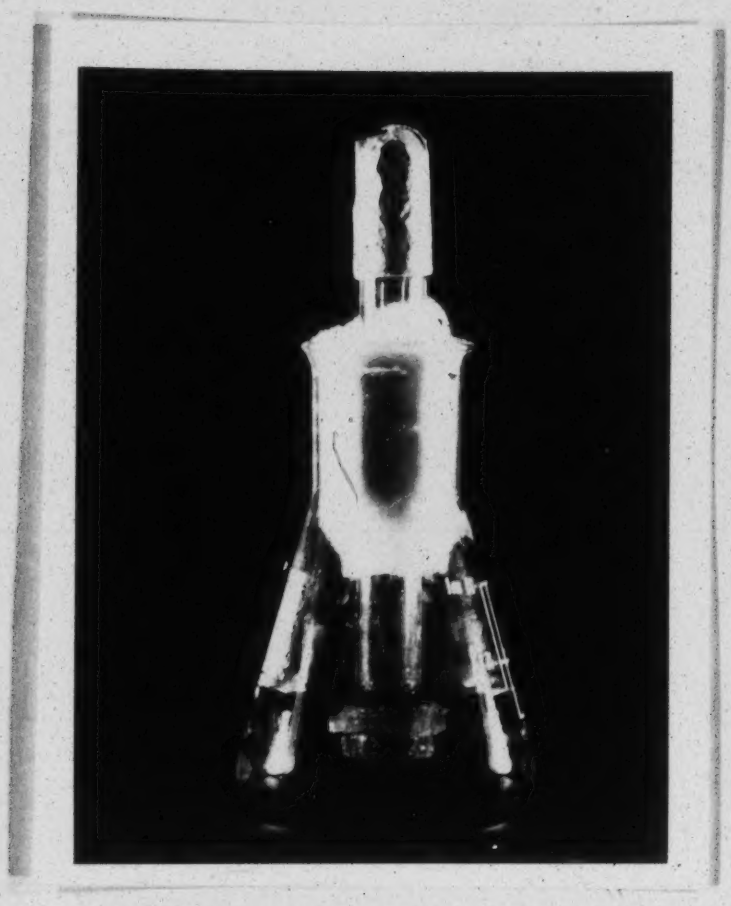


Figure 3 Tissue Culture Flask: Dispersed mouse spleen cells are suspended in tissue culture medium inside an internal tube. The bottom end of the tube is closed off by a dialysis membrane and permits passage of nutrients from the reservoir across the membrane.

monomeric flagellin (MON; mol wt 40,000) and Fragment "A" (mol wt 18,000), isolated from the cyanogen bromide digest of flagellin (Parish, Wistar *et al*, 1969). All three forms of the flagellar antigen share antigenic specificites but they express different degrees of immunogenicity and tolerogenicity *in vivo* and even more so *in vitro*.

In initial experiments, CBA mouse spleen cell suspensions were cultured *in vitro* for four days in the presence of polymerized flagellin (POL), monomeric flagellin (MON) and Fragment "A". Following the culture period, cells were harvested and the number of antibody forming cells (AFC) was determined (Diener, 1968). The degree of immunogenicity correlated with the molecular weight of the antigen: POL was the strongest immunogen, MON was intermediate in effect, and Fragment "A" was virtually nonimmunogenic (Diener and Feldmann, 1970). The effect of increasing concentrations of POL on the primary *in vitro* response was studied (Diener and Armstrong, 1967; 1969). CBA mouse spleen cells were cultured for four days in the presence of varying concentrations of POL. To control for antigen specificity, immunogenic concentrations of antigens different from *Salmonella adelaide* were added to the same cultures. It was found that a mere tenfold increase in POL concentration would convert an optimal *in vitro* immune response to virtually complete unresponsiveness. This phenomenon of antigen induced unresponsiveness *in vitro* fulfilled the criterion of tolerance since it was antigen specific as indicated by the normal response to unrelated antigens and furthermore, could be transferred to lethally x-irradiated recipients (Armstrong and Diener, 1969).

The unresponsiveness induced *in vitro* with supraimmunogenic

concentrations of POL appeared analogous to the phenomenon of *in vivo* high zone tolerance referred to earlier. Because of the antigen dose relationship for tolerance induction, it had been suggested that the direct interaction of immunocompetent cells with more than a critical number of antigen molecules results in tolerance. The quality of the antigen POL, with available smaller antigenic units (MON and Fragment "A") prompted Diener and Feldmann (1970) to test this hypothesis. When CBA spleen cells were incubated in tissue culture with variable dose ranges of MON or Fragment "A" for six hours and were subsequently challenged with an immunogenic concentration of POL, MON proved significantly less effective in its tolerance inducing capacity than POL, while Fragment "A", an excellent tolerogen *in vivo*, entirely failed to induce tolerance within the wide concentration range tested *in vitro*. The conclusion was that non-polymeric antigens such as Fragment "A" must require a mediating mechanism not present in tissue culture. Antigens such as POL, bearing repeated antigenic determinants, however could become attached securely to the cell surface by virtue of a serial combination of repeated identical antigenic determinants with a large number of recognition sites randomly arrayed over the cell surface (Diener and Paetkau, 1972). Indeed the studies of Feldmann (1971) using the dinitrophenyl group (DNP) conjugated to *Salmonella* flagellin polymer, conclusively demonstrated that at least two groups per monomeric unit of flagellin were required for this hapten-carrier unit to be tolerogenic. In other words, high hapten or ligand density permits closely spaced bonding to antigen sensitive receptors on the surface of the immunocompetent cell. Since, in contrast to the above *in vitro* studies,

monomeric antigens most easily induce specific unresponsiveness *in vivo* (Ada and Parish, 1968) it was reasoned that (1) microanatomical or (2) humoral factors were operative in the intact animal but absent under conditions of cell culture.

Studies of Dresser and Mitchison (1968) in adult animals, and Sterzl and Trnka (1957), and Sterzl (1966) in newborns had detected transient antibody production *in vivo* followed by an unresponsive state. This work suggested that the relevant humoral factor might indeed be antibody which could facilitate the mechanism proposed for POL by interlinking MON antigenic units. Given that the recognition antibody structures on the cell participate in this framework, the analogy becomes complete since cell surface recognition sites could become interlinked to the extent required for tolerance induction. Thus a lattice of antigen and antibody could build up at the surface of immunocompetent cells. The degree of linearity of such a complex would be determined by the molar ratio of antigen to antibody. The conditions for the formation of linear antigen-antibody complexes (simulating the linear structure of POL) occur most readily under conditions of slight antigen excess (Pauling, 1940).

These hypothetical considerations (Diener and Feldmann, 1970) are illustrated schematically in Figure 4 and proved verifiable in culture. Spleen cells were cultured with MON or Fragment "A" and various dilutions of specific hyperimmune antiserum for six hours and were subsequently challenged with an immunogenic concentration of POL. As predicted (Figure 5) there was a distinct antigen-antibody concentration ratio which rendered immunocompetent lymphoid cells

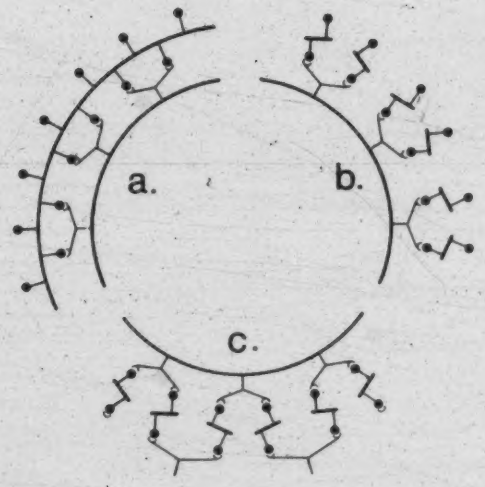


Figure 4 Schematic drawing illustrating different ways of interaction on the cell surface between antigen or antigen and antibody, with antigen recognition sites.

(a) Attachment to the recognition sites of a polymeric antigen.
(b) Attachment to the recognition sites of a monomeric antigen.
(c) Attachment to the recognition sites of a monomeric antigen in the presence of specific antibody.

Note: In situation (a) and (c) antigen-recognition sites become interlinked by the antigen alone or by antigen-antibody complexes, respectively.

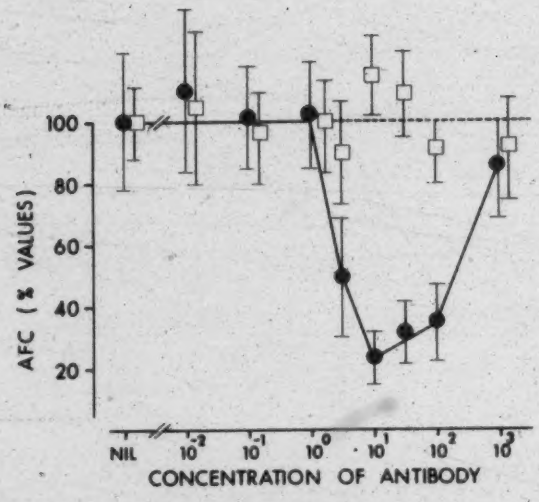


Figure 5 Induction of tolerance to an immunogenic concentration of polymer of *S. adelaide* flagellin (POL) *in vitro* in the presence of varying concentrations of specific antibody. Spleen cells were incubated *in vitro* for 6 hours in the presence of antigen and antibody, followed by challenge *in vitro* for 4 days with POL. Each value represents the mean of 8 cultures ±S.E.M.

Anti-POL: Antibody to polymerized flagellin.
AFC: Antibody forming cells.
● Preincubation with POL and anti-POL followed by culture with POL.
□ Preincubation with sheep erythrocytes followed by culture with the same antigen.

Data reproduced with permission from J Exp Med (Diener & Feldmann, 1970).

unresponsive (Diener and Feldmann, 1970). Evidently tolerance induction *in vitro* results from the direct interaction of immunocompetent cells with antigen at the level of surface recognition sites. The conditions which determine tolerance or immunity depend on the degree of interlinking of recognition sites by antigen. Interlinking beyond a certain level results not in immunity but tolerance. The conditions of tolerance induction are most easily achieved with polymeric antigens because they bear repeating antigenic units which can attach to large numbers of specific recognition sites. Monomeric antigens may be rendered "polymeric" by interlinking them with bivalent antibody. With initial attachment of monomeric antigen to recognition sites, antigen and antibody at defined concentration ratios will readily form a lattice or complex on the surface of the lymphocyte. This focussing mechanism permits interlinking of receptors sufficient for tolerance induction.

5. Experimental Allograft Tolerance *In Vivo*

The permanent survival of allografts has been extraordinarily difficult to achieve across a major histocompatibility barrier in adult experimental animals, unless large doses of immunosuppressive agents are administered continuously, throughout the life of the graft. Specific immunosuppression and/or allograft tolerance induction in adult animals remains an elusive goal. There are, of course, the already described experiments of Stuart, Saitoh *et al* (1968) and French and Batchelor (1969) involving rat renal allografts enhanced with specific alloantiserum. It appears that the two criticisms applied to these models, namely the special complement system in the rat and the fact that grafts are semi allogeneic (i.e. F_1 to parent) may be

pertinent. Renal allografts, for example, cannot be enhanced permanently in an outbred species such as the dog (Williams, 1973). In work with small laboratory animals Monaco, Wood *et al* (1966) and Lance and Medawar (1970) have been able to establish tolerance to rat and mouse skin allografts by ALG treatment and establishment of lymphoid chimerism. Notably absent, however, are reports dealing with adult-induced transplantation tolerance in species other than the rat, without concomitant lymphoid chimerism and using other organs. A singular exception exists in the case of porcine liver allografts (Calne, White *et al*, 1969). It has been suggested in this regard, that allografts of pig liver survive well because the liver provides a good source of tolerogenic transplantation antigens and, possibly, that the normally reversed architecture of the pig lymph node, reminescent of the early stages of development of the mouse lymph node (Williams, 1966), may facilitate tolerance induction.

D. Justification for Thesis Research

The literature survey presented, descriptive of antigen, antibody and lymphocyte interactions in tolerance induction, is controversial, especially with regard to the *in vivo* phenomenon of specific allograft survival. For the purpose of further exploring tolerance induction *in vivo* it is suggested that:

1. All animals specifically bearing an allograft of long duration be considered operationally tolerant. The mechanics of this achievement are undoubtedly subtle and complex. The observed phenomena of humoral antigen, antigen-antibody

complex, antibody mediated immune suppression and central failure of lymphocyte reactivity may all obtain. The data are not necessarily individually exclusive. Variable observations reflect, most probably different assay systems, each bearing singular focus.

2. Allograft tolerance is best studied within the well defined histocompatibility systems of the mouse.

3. Skin allografts, while they have served immunobiologists well, may not be an optimal graft choice for tolerance studies *in vivo*. Skin allografts are difficult to functionally evaluate, and constitute an indeterminate antigen load as they contain significant numbers of lymphocytes and other cells. A skin allograft therefore represents the introduction of allogeneic lymphoid cells (with the possibility of local graft-versus-host interactions) plus skin specific and H-2 transplantation antigens. Exposed to the microbial environment as they are, skin grafts are susceptible to infection, most notably in the irradiated animal.

4. There is a need for a defined allograft system in the mouse which is technically simple and permits functional evaluation. Immunity within such a system (for instance the fetal heart graft model explored in this text) must first be established. Thereafter the mechanisms whereby experimental tolerance may be procured *in vivo* may elucidate the differential function of antigen, antibody and lymphocyte in graft acceptance.

Subsequent chapters describe an allograft model which normally prompts early and specific rejection, but in which allograft acceptance can be established. The nature of this tolerance is explored with various techniques.

CHAPTER II

MATERIALS AND METHODS

The following provides a brief description of the laboratory animals and techniques which were used during the course of experiments described in this thesis. Where appropriate, further explanation is given in the relevant chapter or provided in the Appendix.

A. Mice

CBA/HI (H-2k), Balb/c (H-2d), (CBAxBalb/c)F_1 and C57Bl (H-2b) mice of indicated sex, bred at Ellerslie Animal Farms, University of Alberta, 70-120 days old were used throughout. Pregnant 6-10 week old Balb/c mice (mated to Balb/c males) were used as a source of fetal hearts after 17-19 days gestation. Gestation date was assessed by the vaginal plug method. Animals were housed 5-6 per cage, fed Purina Lab Chow and provided with water *ad libitum*.

B. Tissue Culture

The method of Diener and Armstrong (1969) as recently described by Feldmann and Diener (1971b) was used. Mouse spleen cells were placed on a dialysis membrane sealing off the end of a glass tube which was immersed in an Erlenmeyer flask containing 50 ml tissue culture medium. The culture medium used was a modified Eagle's minimal essential medium

with non-essential amino acids (Grand Island Biological Co., Grand Island, New York) supplemented with 10% fetal calf serum and 100 µg/ml each of penicillin and streptomycin.

C. Cell Suspensions

Mice were killed by cervical dislocation and the spleen or femoral bone marrow was transferred into cold (4°C) Leibowitz medium. Splenic tissue was minced with fine scissors and cells were gently expressed through a stainless steel sieve using a glass pestle. Bone marrow was titrated with a pasteur pipette and cell clumps were allowed to settle out of suspension over a period of 15 minutes. In certain experiments lymph nodes were removed from axillary, cervical, mesenteric and inguinal node regions and were handled in the same fashion as spleen.

D. Cell Counts

Cell counts were performed with a haemocytometer under phase contrast optics except in cases where large numbers of samples were to be counted. For the latter purpose, cell counts were performed with a Coulter Counter Model B (Coulter Electronics Inc., Hialeah, Florida) which was fitted with a 70 µ diameter aperature.

E. Irradiation

Mice were irradiated in a 'Gamma Cell 40' (Atomic Energy of Canada Ltd., Ottawa, Ontario) at an absorbed dose rate of 126 rads per

minute emitted from two 1000 G sources of Cs^{137}. During the γ ray exposure 25-30 mice were housed in a circular plastic container which fits tightly into the drawer of the irradiation unit. Air is supplied to the drawer and container during the irradiation period. This type of irradiation unit and its use is described in detail by Cunningham, Bruce *et al* (1965). Mice received 950R whole body irradiation (Fig. 6).

F. Titrations

1. Hemagglutinating antibody titres were performed using the method of Stimpfling (1961).
2. Cytotoxic antibody titres were performed as described by Terasaki and McClelland (1964).

G. Preparation of Antisera

1. Rabbit Anti-mastocytoma Serum (RAMS)

Adult male San Juan rabbits (Ellerslie Animal Farms, University of Alberta) were given three subcutaneous neck injections of 10^9 washed Balb/C mastocytoma cells at three week intervals. Rabbits were exsanguinated by cardiac puncture 10 days following the last injection. Clotted blood was placed in a 37°C incubator for one hour, in a 4°C cold room overnight and the serum was pipetted off the cell pellet following centrifugation at 2000 rpm for 10 minutes. Serum derived in this manner was used whole or as a source of rabbit anti-mastocytoma IgG.

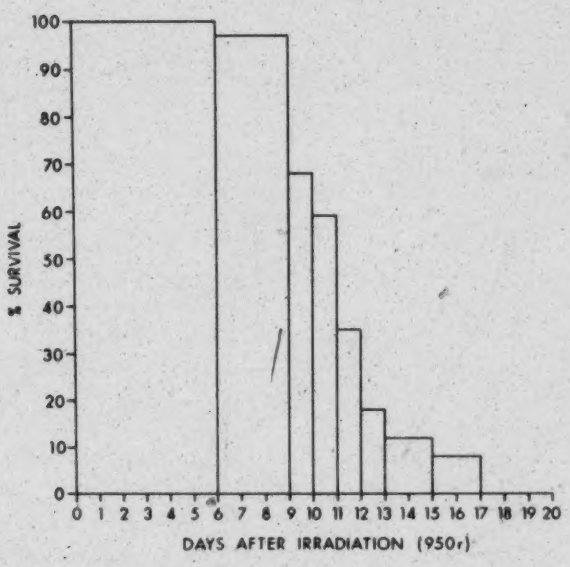


Figure 6 Survival of 66 adult CBA/HI mice after 950 rads whole body irradiation. None of the animals survived longer than 17 days post-irradiation.

2. CBA Anti Balb/c Serum

Adult male or female CBA/HI mice were given three intraperitoneal injections of 10^8 washed Balb/c spleen cells three weeks apart and were bled by cardiac puncture 10 days following the final injection. Serum was handled as RAMS.

3. Horse Anti Mouse Thymocyte Serum (HAMTS)

The Medical Research Council of Canada kindly made available 300 ml of active HAMTS. This was prepared by the Institut de Microbiologie et d'Hygiene de l'Universite de Montreal, Laval-des-Rapides, Quebec.

H. Preparation of IgG

1. Rabbit IgG

Gamma globulin was precipitated from serum by the addition of 30% ammonium sulfate at room temperature. The precipitate was dissolved in deionized water and reprecipitated three times. Finally the precipitate was dissolved in phosphate buffered saline (PBS) in approximately one half the original serum volume. This was applied slowly, by means of a motor driven syringe, onto a Sephadex G200 column equilibrated with PBS at 4°C. The column was equipped with an automatic fraction collector and ultraviolet detector at 280 mμ to determine protein concentration. The second protein peak was taken as IgG and was dialyzed overnight against deionized water in the presence of chloroform at 4°C and subsequently freeze dried. IgG was reconstituted in PBS to approximate the starting serum volume; 1% bovine serum albumin (BSA) was added to

prevent freeze thaw denaturation.

2. Horse IgG

The procedure for rabbit IgG preparation was followed with certain exceptions. Ammonium sulfate precipitates were dissolved in PBS instead of deionized water. During Sephadex G200 separation, merthiolate ($1/10^6$) was present to prevent bacterial growth. The IgG fraction was reprecipitated three times with 50% ammonium sulfate in PBS after Sephadex G200 separation. This removed merthiolate. The final precipitate was dissolved in a serum equivalent of PBS. BSA (1%) was again added to stabilize the protein content.

3. Mouse IgG

Ten ml of mouse serum were applied directly to the Sephadex G200 column. The second protein peak was again collected and reprecipitated three times with 50% ammonium sulfate in PBS. The final volume was adjusted to approximate the starting serum volume.

I. Cell Separation by Velocity Sedimentation

1. Theory of Velocity Sedimentation

Velocity sedimentation is a technique which can be used to characterize dispersed cell populations on the basis of size. Cells may be considered as approximately spherical particles which obey Stokes' law, which defines the terminal velocity of such a particle suspended in fluid:

Stokes' Law

$$s = \frac{2gr^2 (d_c - d_f)}{9\eta}$$

where

s = final velocity
g = force of gravity
r = radius of the particle
d_c = density of particle
d_f = density of fluid
η = coefficient of viscosity of the fluid medium

According to this formula then, if cells are subjected to unit gravity in a medium less dense than all cells, settling will occur toward the bottom of the sedimentation vessel. Viscosity of the fluid will exert a drag force on falling cells and thus decrease the rate at which they settle. If the difference between the density of cells and fluid medium ($d_c - d_f$) is relatively constant for the gradient used, the rate of settling is determined most significantly by the radius^2 of the cell. In other words, velocity of fall is most dependent on cell size. Cells of large size will fall and reach the bottom of a sedimentation vessel most quickly. If, however, serial fractions are removed from the bottom of the vessel before this, different cell populations will be characterized by their unique sedimentation velocities, corresponding to cell size.

2. Components of the System

The system used is pictured in Figures 7 and 8 and is essentially

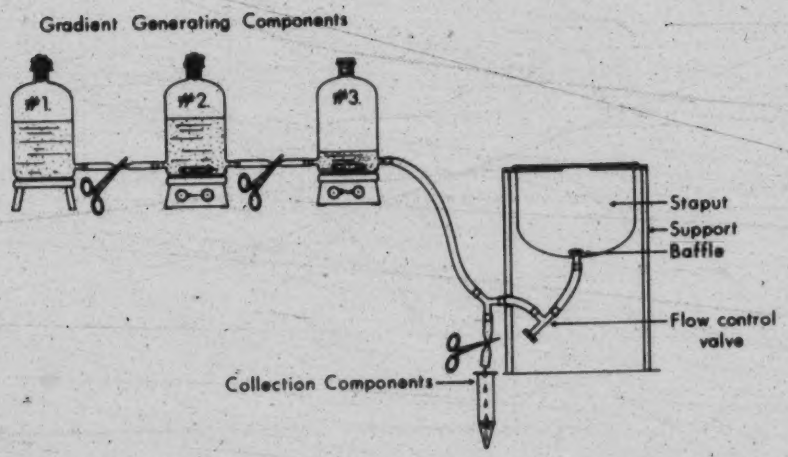


Figure 7 Schematic illustration of the "staput" apparatus for separation of dispersed cells by velocity sedimentation.

Figure 8 Actual photograph of velocity sedimentation apparatus in 4°C cold room, prior to gradient formation.

that described by Peterson and Evans (1967) and later used by Kraft and Shortman (1972) and Armstrong and Kraft (1973).

a. Baffle - stainless steel hemisphere with three equidistant legs on the undersurface; functions to prevent turbulence of fluid moving in and out of the staput vessel and evenly distributes fluid flowing up through the staput port.

b. Bottles - three sterilizable bottles connected as shown. The bottles contain 400 ml 30% FCS, 400 ml 15% FCS and 50 ml 5% FCS in phosphate buffered saline (PBS) from left to right respectively. The third bottle is tightly corked to maintain a constant volume. Four clamps are positioned as shown to regulate fluid movement. The bottles are connected by short lengths of silastic tubing and a flow control valve is positioned between the solution bottles and the staput vessel. The second and third bottles are positioned on motor driven stirrers and contain teflon coated stirrer bars. All bottles are kept at the same level, which is above that of the staput.

c. Staput - an autoclavable glass cylinder 16.5 cm in diameter which tapers to a cone. The bottom of the cone is fitted with a vertical outlet port; the top of the cylinder is fitted with a lip, permitting support of the vessel inside a wooden box with a hole in the top as shown.

d. Tubes (fraction collection) - 15 ml collection tubes are required along with several graduated cylinders or flasks.

e. Y-piece - glass component positioned close to the control valve. The vertical component of the Y-piece permits fraction collection and is connected to a piece of tubing into which a pasteur pipette is inserted. The tubing is clamped until fraction collection when the tubing that connects with the third bottle is clamped shut.

3. Operation of the System

a. General

The system shown merely provides a means of generating a non-linear, shallow density gradient comprised of 3 to 30% FCS in PBS. The three connected bottles contain differing concentrations of FCS as mentioned above and the gradient is removed by gravity flow from the third bottle into the staput at a rate of approximately 30 ml/min. A total of 10^7 cells or less per ml are placed in each staput in a solution of 3% FCS in PBS. Once the gradient is formed cells are sedimented at 4°C for variable periods (2.5 hours in experiments in this text) after which 15 ml fractions are collected from the bottom of the staput. All FCS used in the system is centrifuged at 500 G for 20 min before use to remove particulate matter.

b. Specific

(i) The system is arranged as shown in Figure 9.

(ii) Bottles #1, 2 and 3 are filled with 30% (400 ml), 15%

(400 ml) and 5% (50 ml) FCS in PBS respectively. Clamps are placed as noted and Bottle #3 is tightly stoppered. Stirrers operative.

(iii) Dispersed mouse spleen cells in 3% FCS in PBS (concentration $5\text{-}10\times10^6$ cells/ml) are poured into the bottom of the staput vessel.

(iv) Clamps #1 and #2 are released. The flow control valve is opened and time is noted. The rate of fluid influx monitored at 25-30 ml/min.

(v) Time records are kept:

1 - when fluid level reaches top of staput cone

2 - at beginning of fluid efflux (collection)

3 - when falling fluid level reaches the top of the cone

4 - when collection of fluid complete.

4. Determination of Cell Velocity

If cells start to fall from the top of the fluid gradient and if the time elapsed before collection of fractions is known, a sedimentation velocity can be calculated for each fraction. Knowing the vertical depth occupied by one fraction (depth per fraction) will permit calculation of an s value for each fraction. The following formula is used on a computer program (Kraft, 1972) which yields a printout with an s value for each fraction:

$$s = \frac{60\ (X_E - X)\ D}{T_s + \frac{T_c}{X_T}\ (X-1)}$$

where

s = sedimentation velocity in ml/min

X_E = effective number of fractions collected

X = actual fraction number, e.g. fraction number 3

D = depth per fraction

T_s = staput standing time (hours)

T_c = collection time (hours)

X_T = total number of fractions collected

In this formula

$$X_E = X_T - Y \qquad \text{where } Y = \frac{n}{2} - \frac{1}{2}$$

$$\text{with } n = \frac{\text{sample volume or loading volume}}{\text{fraction volume}}$$

T_s = the time from when gradient liquid (on influx) reaches the top of the staput cone to the time when fluid removal begins, i.e. the time that fluid occupied the vertical section of the staput PLUS $\frac{1}{2}$ of the cone time. The cone time represents the time fluid spends filling the cone until the vertical portion of the staput begins to fill PLUS the time fluid, leaving the vessel, spends emptying from the base of the vertical cone section until the cone is empty.

T_c = time when fluid removal commences to the time when the top of the gradient fluid reaches the base of the vertical section of the staput (i.e. the time required to empty the vertical section).

This formula takes into account the fact that the staput represents not just a cylinder but a cylinder with a cone attached.

Once the gradient is formed and the staput is full, the cells in suspension initially introduced into the vessel are no longer at the top of the fluid layer. Cells will have fallen during staput filling and will no longer occupy a narrow starting band. Factors X_E and D attempt to determine cell position. Since cells continue to fall during collection, the factor $\frac{T_c}{X_T}$ (X-1) is included in the formula to account for this time factor.

J. The Brunner Assay

Cellular immunity induced *in vivo* and *in vitro* was studied with the aid of an *in vitro* system measuring the cytotoxic effect of sensitized lymphocytes on ^{51}Cr labelled target cells. Inbred female mice of Balb/c strain served as donors of mastocytoma cells. The mastocytoma tumor, originally obtained from Dr. F. Wunderlich, National Institute of Health, Bethesda, Maryland, USA, was maintained by serial passage in ascitic form. Tumor cells were suspended in Leibowitz medium containing 10% FCS (L/FCS) and the suspensions were adjusted to $8\text{-}10\times10^6$ viable cells/ml. To 0.4 ml of cell suspension 0.05 ml of a solution of radioactive sodium chromate (^{51}Cr sodium chromate, 1 mCi/ml, specific activity 100-400 μ Ci/μg of chromium; Radiochemical Centre, Amersham, England) was added, and the cells were incubated for 30 minutes at 37°C. The cells were then washed three times by centrifugation in L/FCS and adjusted to 10^5 viable cells/ml. Volumes of 0.5 ml of reaction mixtures containing a standard number of target cells (1×10^4) and 1×10^6 of either sensitized or normal lymphocytes were

placed in 5 ml Falcon tubes. After nine hours residence in an incubator at 37°C, 1.0 ml of L/FCS was added to each tube, followed by centrifugation at 450 G for 7.5 minutes. Taking care not to disturb the sediments, 1.0 ml volumes of the supernatant fluids were then removed with a 1 ml pipette. These samples were placed in 74x12 mm round bottomed glass tubes for measurement of radioactivity in a well-type scintillation counter (Packard Tri Carb Scintillation Spectrometer model 3002, Downersgrove, Illinois, USA).

Calculations were based on the total amount of radioactivity released into the supernatant relative to the total amount of activity present. Total activity was obtained by counting both supernatant aliquots and the tube containing the precipitate and remaining supernatant.

1. For each sample the percentage of ^{51}Cr release was calculated according to the formula:

$$\% \text{ supernatant release} = \frac{\text{test supernatant release - control release}}{^{51}Cr \text{ available - control release}} \times 100$$

where

test supernatant release = ^{51}Cr release in 1 ml of the supernatant tested

control release = ^{51}Cr release in 1 ml supernatant of control sample; the control sample consisted of Balb/c, or nonreactive, cells incubated with ^{51}Cr labelled Balb/c mastocytoma cells over 9 hours.

^{51}Cr available = the ^{51}Cr label present in 0.67×10^4 labelled mastocytoma cells alone subjected to 9 hours incubation.

The remaining precipitate, after withdrawal of 1 ml supernatant, was also counted.

In a trivial example:

	Supernatant Count	Precipitate Count
(1) CBA sensitized to Balb/c + ^{51}Cr labelled mastocytoma cells	60	40
(2) Balb/c cells + ^{51}Cr labelled mastocytoma cells	10	100
(3) ^{51}Cr labelled mastocytoma cells	80*	35†

*1 ml sample was taken after 9 hours mastocytoma cell incubation without precipitation

†0.5 ml sample remaining after 9 hours mastocytoma cell incubation without precipitation

Applying the formula to the test sample of the sensitized CBA + mastocytoma mixture

$$\% \text{ release} = \frac{60 - 10}{80 - 10} = \frac{50}{70} = 71.4\%$$

2. However pipetting errors are evident in that supernatant and precipitate counts totalled, are not equal throughout. One can, therefore, for each two values of supernatant and precipitate, determine the percentage of ^{51}Cr released into the supernatant.

$$\text{Supernatant Total Release (STR)} = \frac{(\text{Supernatant Release})\ 1\frac{1}{2}}{\text{Supernatant Release} + \text{Precipitate Release}} \times 100$$

*The $1\frac{1}{2}$ factor is introduced since the supernatant counts are done on 1 ml of a 1.5 ml mixture of supernatant and precipitate.

For the figures given then

$$\text{STR} = \frac{(60)\ 1\frac{1}{2}}{60 + 40} = \frac{90}{100} \times 100 = 90\%$$

These can be calculated for each test tube.

3. If the control experiments (mixtures of Balb/c or unresponsive cells mixed with ^{51}Cr labelled mastocytoma cells) are considered as baseline figures, a Converted Total Release (CTR) can be determined for test samples using the following formula.

$$\text{Converted Total Release (CTR)} = \frac{\text{STR (test sample)} - \text{Control STR}}{100 - \text{Control STR}} \times 100$$

or, in the example noted:

$$\text{CTR (test)} = \frac{90.0 - 13.6}{100\text{-}13.6} = \frac{76.4}{86.4} \times 100 = 86.1\%$$

Expression of Results as % Supernatant Release; % Supernatant Total Release and Converted Total Release reflect different means of expressing the release effected by test cells in the Brunner assay. The first formula is most simple and does not take into account pipetting errors; the formula for Supernatant Total Release individualizes the results for

each sample without considering control effect. The final value, Converted Total Release regards the release effected by control or nonreactive cells as a baseline value and compares all samples to this baseline.

K. The Hellström Assay

The technique used was essentially that described by Hellström, Hellström *et al* (1971). Mice derived from the same strain (Balb/c) that contributed fetal hearts for allotransplantation were used as sources of fibroblast target cells. These fibroblasts, derived from footpad tissue or fetal lung were maintained in tissue culture in Waymouth's solution containing 23% FCS (W/FCS). Subsequent to trypsin removal and reconstitution in W/FCS, fibroblasts were plated into the wells of 2040 Falcon Microtest II plates (96 wells/plate) at a constant number per well in a given experiment. Approximately 200 fibroblasts in 0.1 W/FCS were plated, of which approximately 100 became adherent to the bottom of each well. After 24 hours incubation at 37°C this medium was discarded and diluted serum in whole Waymouth's solution (from tolerant or normal mice; quantity 0.05 or 0.1 ml) was added for a 30 minute incubation period at 37°C. This serum was then discarded and then 0.1 ml of a spleen cell suspension containing 1×10^5 cells in Waymouth's medium was added to each well. After a further 30 minute incubation an additional 0.1 ml of W/FCS was added to each well and the plates were incubated overnight at 37°C. Thereupon all medium was decanted. Wells were rinsed twice with Leibowitz solution and cells were stained with crystal violet. Crystal violet was washed

away by rinsing with water and the plates were dried in the incubator at 37°C for approximately two hours. The plates were subsequently examined under a microscope for the number of remaining fibroblasts per well.

CHAPTER III

DEVELOPMENT OF THE EXPERIMENTAL MODEL: TRANSPLANTATION OF THE FETAL MOUSE HEART

A. Introduction

The initial difficulty in developing a model for studying transplantation tolerance was the choice of an allograft. Two common graft systems have been used in the laboratory, namely skin grafts in rodents and renal allografts in rats and dogs. Each of these have particular disadvantages. Skin grafting, while technically easy, does not permit accurate evaluation of graft function and does not have a precise rejection end point. Renal allografts, on the other hand, can be followed functionally but are tedious to implement, involving microvascular anastomoses, and are thus fraught with technical difficulties. They are further of dubious value in transplantation studies since long-term kidney survival can be obtained in rats allografted across the major (Ag-B) histocompatibility barrier without immunosuppressive treatment (Salaman, 1971; Daniller, 1971). Because of the obvious need for an allograft system in small animals which was both technically simple and rapid, while permitting functional evaluation, attention was turned to fetal cardiac allografts as a transplantation model. Electrocardiography, a precise measurement of the persistence of functioning cardiac tissue, would seem a convenient means of evaluation.

Free grafting techniques have been used to study transplanted whole hearts in the hamster (Poor, 1957), chick embryo (Katzberg, 1959), platyfish (Weinstein, 1960) and mouse (Conway, Griffith *et al*, 1958; Fulmer, Cramer *et al*, 1963). Only recently, however, has the model received attention as a means of monitoring transplantation immunity (Judd, Allen *et al*, 1969). These authors transplanted newborn and fetal hearts of indeterminate age and assessed the effect of an anti-lymphocyte serum to prolong visual pulsatile activity. Further work in developing this system seemed cogent since the fetal heart bears transplantation antigens (Schlesinger, 1964; Simmons and Russell, 1966), is tolerant to hypoxia and conveniently small to work with.

B. Technique of Cardiac Allografting and Subsequent Evaluation

The original technique of neonatal or fetal heart transplantation in the mouse, as described by Fulmer, Cramer *et al* (1963) was modified. As shown in Figure 9, hearts are removed from fetal mice *in utero*, after 16-18 days gestation by gentle, blunt dissection under a stereomicroscope. The beating hearts, which measure approximately one millimeter in diameter are placed in cold (4°C) Leibowitz solution. Adult recipient animals are anesthetized by the intraperitoneal injection of 6×10^{-2} mgm/gm mouse sodium pentobarbital (Diabutal, Diamond Laboratories, Des Moines, Iowa) diluted in Leibowitz medium. Using a tuberculin syringe with attached 26 gauge needle, 0.1 ml of Leibowitz solution is injected subcutaneously into the anterior aspect of the mouse ear, raising a small fluid filled bleb. This bleb is opened along its

Figure 9 Technique of cardiac allografting in the mouse.

1 - subcutaneous ear pocket formed by injecting a small amount of saline

2 - bleb thus formed is opened along outer margin

3 - insertion of 18-day gestation fetal mouse heart

4 - graft in place.

(Jirsch, Kraft *et al*, 1973)

outer margin with sterile scissors and the fetal heart is introduced with forceps into the subcutaneous space. Here the small graft is nourished by surrounding tissue fluid and serum until capillaries begin to grow into marginal myocardial tissue some days later.

Allograft cardiac function following transplantation is evaluated primarly by electrocardiography with visual confirmation of graft pulsation through the thin overlying skin of the external ear (Figure 10). The limb leads of a standard ECG machine (Electronics for Medicine, Model DE 3562, White Plains, New York, USA) with high sensitivity and rapid paper speed (to accommodate the mouse heart rate of 400-600 beats per minute) are attached to animals momentarily anesthetized with methoxyflurane (Penthrane, Abbott Laboratories, Montreal, Canada) by means of small spring-loaded copper clips (Technical Services Workshop, University of Alberta) (Figures 11 and 12). A smaller clip is attached to the periphery of the mouse ear containing the fetal heart graft and is connected proximally to the V-lead or search electrode. In this manner, the electrical activity generated by both the adult host heart and that of the heterotopic fetal graft are recorded simultaneously as two distinct sets of independent rhythmical electrical activity (Figure 13). Whether or not allograft electrical activity is present provides a stringent and precise index of graft survival.

C. Results of Grafting

In a series of 20 adult CBA mice which received Balb/c fetal heart allografts, 19 of 20 mice began to show electrical activity on the

Figure 10 Fetal heart graft as visualized with magnification. The small discolored area represents the graft *in situ*. At higher magnification pulsatile activity is apparent through the overlying skin.

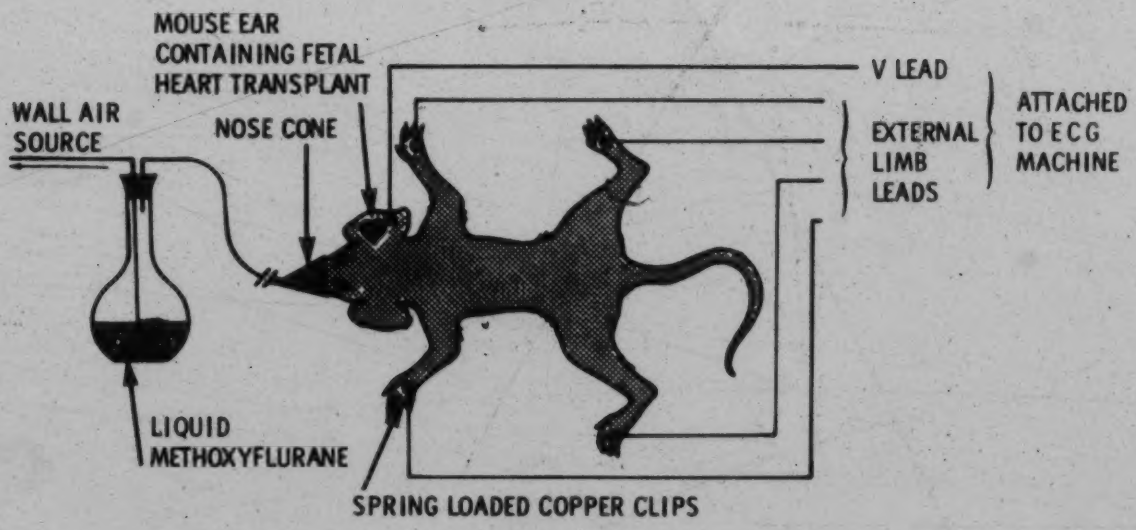


Figure 11 Schematic representation of method of anesthesia and electrocardiography permitting evaluation of cardiac graft activity.

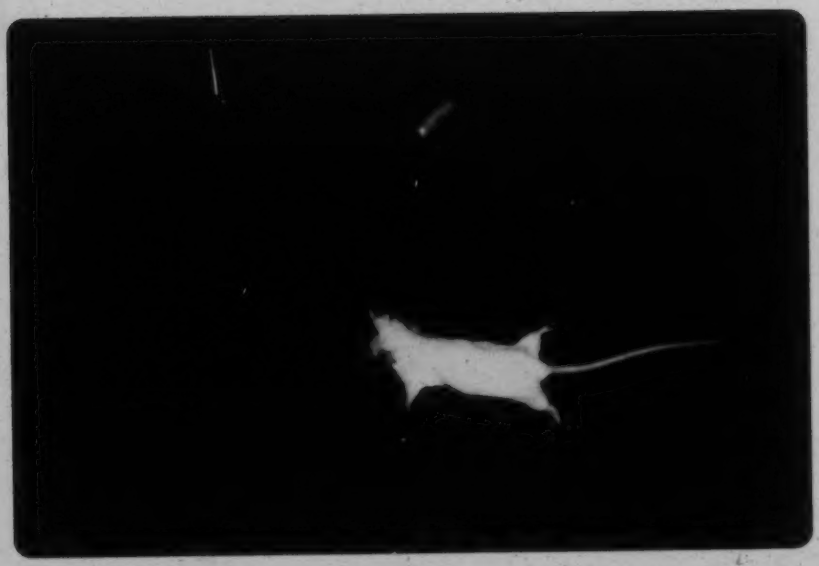


Figure 12. Photograph of an anesthetized mouse with spring loaded copper clips attached to the extremities (limb leads) and a smaller clip (V lead) attached to the relevant ear containing a fetal heart graft.

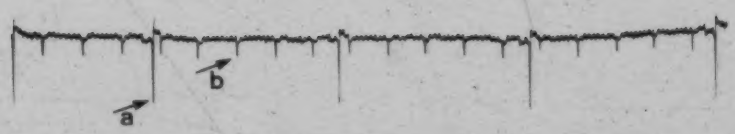


Figure 13 Typical electrocardiogram obtained from a cardiac allografted mouse

a - electrical activity generated by the heterotopic graft

b - adult heart electrical activity

fourth day following transplantation (Figure 14). This activity fell precipitously on the seventh day after allografting due to immune destruction of the heart, reflected histologically thereafter as a diffuse mononuclear cell infiltrate with gross disruption of myocardial fibers (see Figures 15-17). Second Balb/c grafts inserted in either the same or opposite ear of these mice reflected an anamnestic or memory response (Figure 14). Thus, only 50% of second grafts ever established electrical activity and, in these, rejection was more rapid with major loss of electrical activity on the fifth day post-allografting. Fetal hearts transplanted into twelve identical strain mice developed visible pulsation and detectable electrical activity in 11/12 instances. These grafts did not reject and survived indefinitely. Visual monitoring of graft electrical activity was most rewarding in these cases, for postoperative tissue swelling and bruising made direct observation of cardiac activity difficult in the first week after surgery. Syngeneic grafts did not grow to any significant extent, most probably due to the absence of a work load, but pulsation and electrical activity persisted indefinitely.

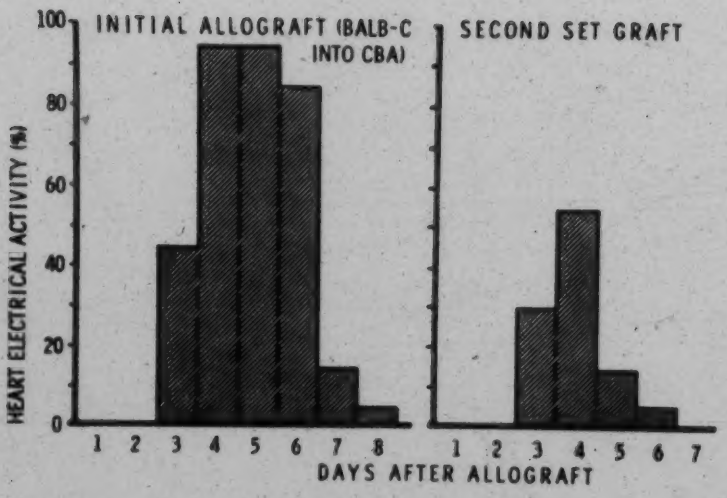


Figure 14 Primary and secondary cardiac allograft survival and rejection. See text.

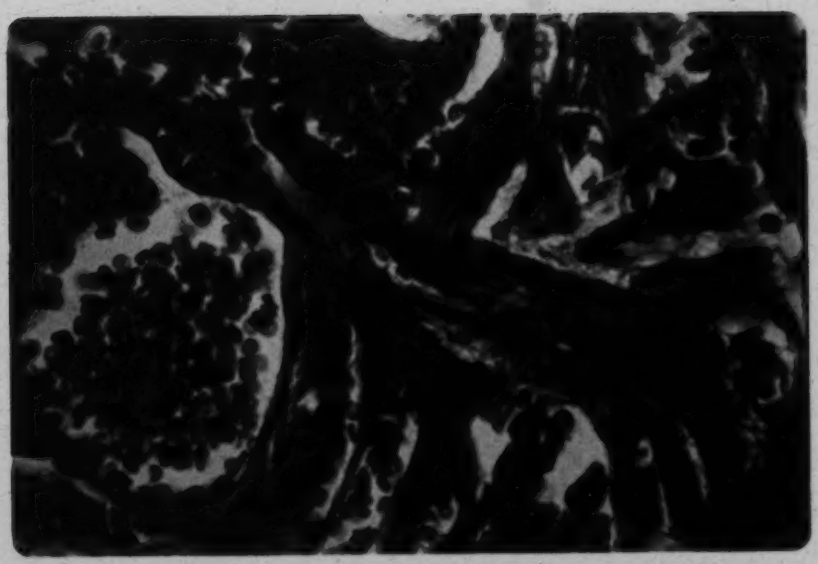


Figure 15 Normal histology of a syngeneic fetal heart graft after five months heterotopic residence (Balb/c fetal heart into adult Balb/c mouse ear). Magnification ×160; Gomori's trichrome stain.

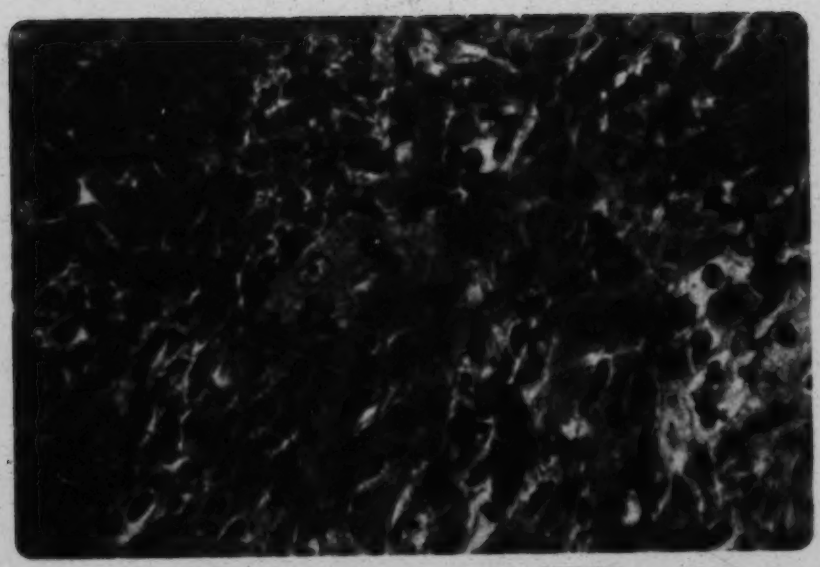


Figure 16 Representative histologic section of a Balb/c allograft fetal heart transplanted into the subcutaneous ear tissue of an adult CBA mouse. This section (magnification ×160; Hematoxylin & Eosin stain) reveals a diffuse cellular infiltrate with near total destruction of myocardial tissue ten days after transplantation.

Figure 17 Representative histologic section - a Balb/c fetal heart resident in an adult CBA mouse ear after four months. Tolerance to the fetal heart was induced by methods described in Chapter VI. Note retention of normal myocardial architecture and absence of a significant cellular infiltrate. Magnification ×100 Gomori's trichrome stain.

CHAPTER IV

IMMUNE EXPRESSION AND HETEROTOPIC TRANSPLANTATION OF THE MOUSE HEART

The precision with which allograft hearts were rejected suggested that this was indeed a sensitive transplantation model. It was decided to monitor the development of cell mediated and humoral immunity in allograft recipients at various intervals following graft insertion. This would compare the functional sensitivity of the heart allograft model with the expression of cell mediated immunity *in vitro* (Brunner, Mauèl *et al*, 1968) and the humoral expression of graft directed antibodies.

A. Methods

1. *In Vitro* Assay of Cell Mediated Immunity

The Brunner assay has been described. Spleen cells from mice which had received either intravenous injections of Balb/c spleen cells; single or multiple Balb/c fetal hearts or adult skin grafts were tested. 5×10^6 CBA spleen cells were incubated with 5×10^4 ^{51}Cr labelled Balb/c mastocytoma cells for nine hours. Subsequent ^{51}Cr release into the supernatant was measured.

Spleen cells were tested from

(a) 3 to 8 heart allografted mice (Balb/c onto CBA), 5, 7, 10 and 14 days following heart transplantation

(b) 6 animals which had received flank skin allografts (Balb/c onto CBA; approximately 0.5 cm^2 area) were tested within 10 days of transplantation

(c) 10 CBA mice which had received two injections of 20×10^6 Balb/c spleen cells 14 days apart

(d) 4 mice were given multiple heart grafts and were tested within 10 days of the final graft.

2. *In Vitro* Assay of Humoral Immunity

The development of humoral immunity was assayed using pooled sera from normal and heart grafted animals at days 4, 7, 10 and 14 after transplantation. The microcytotoxicity test of Terasaki and McClelland (1964) was used. Balb/c lymph node cells, prepared as described in Chapter II, were suspended in Leibowitz solution, washed twice in Leibowitz (450G×7.5 minutes) and resuspended in McCoy's medium at a concentration of 2×10^6 cells/ml. One λ of serum was added to microtest plates (Terasaki microtest plates #3034, Fisher Laboratories, Don Mills, Ontario) with one λ of Balb/c lymph node cells. After 30 minutes (room temperature) 5 λ of rabbit complement (absorbed for one hour against an equal volume of packed CBA erythrocytes) were added to cell wells. Following one hour incubation at room temperature, 5% eosin in distilled water (3.3 λ) was added with subsequent formaldehyde fixation. Percent cell death was then determined on the basis of dye incorporation in six wells tested per serum sample.

B. Results

1. Cellular Immunity *In Vitro*

Representative experiments from each of the above groups are detailed in Appendix, Section 1.

Spleen cells from mice given single, multiple or even repeat allografts to the ear failed to show consistent sensitization. Flank skin allograft recipients showed low but consistent levels of sensitization within 10 days of transplantation. Intravenous immunization of CBA mice with Balb/c spleen cells 14 days apart caused a 60% or greater release of ^{51}Cr. *In vitro* sensitization of CBA spleen cells with Balb/c mastocytoma cells also resulted in a large degree of ^{51}Cr release (49±14%) within a four day incubation period (Figure 18).

2. Humoral Immunity

Results of microcytotoxicity testing are outlined in Table 1. Normal CBA serum was not cytotoxic, but cytotoxic activity developed within four days of transplantation. This persisted with relatively constant activity on days 7 and 10 post-allograft insertion. A marked increase in cytotoxicity was apparent on day 14 when serum, previously undiluted, remained markedly cytotoxic at dilutions of 1/32 and 1/64.

C. Discussion

1. Cellular Immunity *In Vitro*

The ^{51}Cr release assay for cellular immunity was evidently capable of detecting sensitized spleen cells since intravenous Balb/c spleen cells elicited significant ^{51}Cr release. The allografted heart,

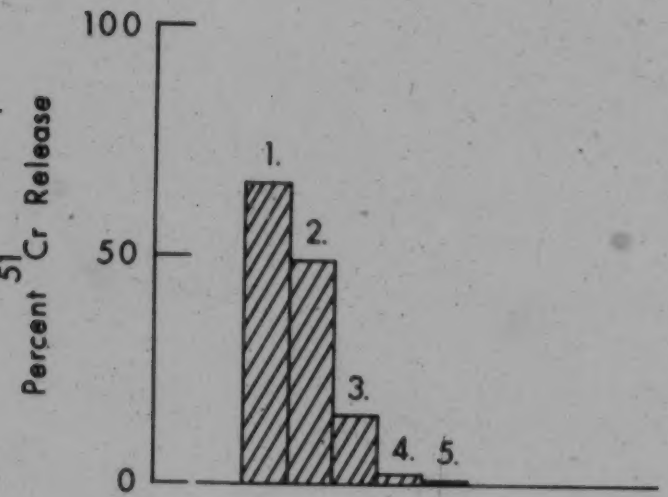


Figure 18 Comparison of sensitization achieved with heart graft transplants with other procedures, as determined by the ^{51}Cr release assay. (1) Two injections of 20×10^6 Balb/c spleen cells 14 days apart; (2) *in vitro* culture of 20×10^6 CBA spleen cells with 5×10^4 Balb/c mastocytoma; (3) flank skin allografts 10 days after transplantation; (4) multiple ear heart allografts 10 days after transplantation; (5) single ear heart allografts 10 days after transplantation.

	Cytotoxicity Value (Each Figure Represents One Well)
Normal CBA Serum	1 1 1
4 Days Post Balb/c Graft	6 6 6 6 6 4
7 Days Post Balb/c Graft	4 6 6 6 6 6
10 Days Post Balb/c Graft	6 6 6 6 6 6
14 Days Post Balb/c Graft*	8 8 8

Legend: 1 - no cytotoxicity
4 - cytotoxicity >10%<25% cells
6 - cytotoxicity >25%<90% cells
8 - cytotoxicity >90% cells

*14 day serum diluted to 1/64 still positive (cytotoxicity value = 6)

TABLE 1 - Cytotoxicity of CBA serum for Balb/c lymph node cells in normal and Balb/c allografted animals. At intervals following transplantation whole sera, pooled from four animals sacrificed on appropriate days, were assayed for cytotoxic activity against Balb/c lymph node cells in the presence of rabbit complement.

however, failed to elicit measurable sensitization. These results were confirmed by Kraft (1972) using an immune inhibition technique similar to that described by Hirano and Uyeki (1971). In this assay the secondary *in vitro* response of CBA spleen cells to sheep erythrocytes can be inhibited by the addition of Balb/c cells sensitized against CBA alloantigens. Using mice from the test groups subjected to the ^{51}Cr release assay, no significant inhibition was obtained with spleen cells from cardiac allografted animals. This indicates that the heart graft model is a more sensitive indicator of transplant rejection than two current *in vitro* assays for cellular immunity.

The lack of sensitization with heart allografts could be attributable to antigenic differences between heart tissue and the target cells used in the *in vitro* assays, as suggested by the recent data of Barker, Lubaroff *et al* (1971) or to the relatively small and localized amount of tissue transplanted. If antigenic differences between the sensitizing tissues and target cells do not explain the lack of cellular immunity observed, current assays for cellular immunity may be too insensitive.

2. Humoral Immunity *In Vitro*

Cytotoxic activity in the sera of allografted animals was present four days following transplantation. At the time of overt graft rejection (7 days as assessed by electrocardiography) however, there was no abrupt rise in cytotoxic potential. Two weeks following transplantation marked humoral cytotoxic activity was present in the serum. It is possible that at the time of observed graft rejection,

the majority of cytotoxic antibodies had been absorbed by the allograft, and were not present in sera for assay. In other words evaluation of humoral cytotoxic antibodies did not correlate with graft rejection.

Electrocardiographic evaluation of graft function thus appears to be a more sensitive indicator of transplantation immunity than either (1) an *in vitro* assay for cellular immunity or (2) serial evaluation of cytotoxic graft directed antibodies.

CHAPTER V

EARLY ATTEMPTS AT TOLERANCE INDUCTION AND ENHANCEMENT

A. Antigen-Antibody Injection *In Vivo*

The molecular model for tolerance established by Diener and colleagues *in vitro* suggested that administration of alloantigen and appropriate antiserum *in vivo* deserved trial. For this purpose Balb/c erythrocytes collected in Alsever's solution and washed in PBS, were used as a convenient antigen. An homologous CBA anti Balb/c serum (prepared as described in Methods; HAT 1/1024) was used as an antiserum to administer in serial dilutions after administration of antigen, in an effort to determine whether there was a critical ratio of alloantigen to antibody which would render recipient animals tolerant.

The format of the experiment is depicted in Table 2. CBA adult mice were given intraperitoneal injections of 4×10^6 Balb/c erythrocytes in PBS every second day for one month. To appropriate groups 0.1 ml of log 10 dilutions of antiserum were added thereafter (also injected intraperitoneally). After 30 days of this pretreatment all mice were grafted with a Balb/c fetal heart in standard fashion. In brief, none of the allografts survived longer than seven days; the majority were rejected within five days of grafting or did not develop graft electrical activity at all. This was not altogether unexpected. The differential mechanisms of antigen and antibody

Group	Intraperitoneal Antigen q2d	Reciprocal Titer (HAT) of of Homologous Antiersum Administered Thereafter	Animals Per Group
A	4×10^6 Balb/c RBC	1000	5
B	"	100	5
C	"	10	5
D	"	1	5
E	"	10^{-1}	5
F	"	10^{-2}	5
G	"	10^{-3}	5
H	"	No antiserum	5
J	No antigen	100	5

TABLE 2 - Antigen-antibody pretreatment of CBA mice. Groups of adult mice were given separate intraperitoneal injections of washed Balb/c erythrocytes and serial dilutions of CBA anti Balb/c antiserum (Hemagglutination Titer = HAT) every second day for one month prior to insertion of a Balb/c fetal cardiac allograft. Control groups received, respectively, antigen (erythrocytes) but no antiserum and a representative dilution of antiserum without erythrocytes.

capture *in vivo*, with the problem of non-uniform dispersal of either, likely precluded the events possible *in vitro* and resulted in sensitization of recipients.

B. Transplantation Antigen *In Vitro*

The availability of a small quantity of purified Balb/c H-2 antigen (courtesy of Dr. S. Nathenson, Bethesda, Maryland) prompted *in vitro* testing analogous to the POL-anti POL experiments. To 20×10^6 washed CBA spleen cells in 1 ml of MEM/10% FCS, 0.1 ml of the protein transplantation antigen was added. Serial dilutions of rabbit anti Balb/c antiserum (prepared as described in Methods) were added to appropriate cultures as depicted in Table 3. After 24, 48 or 72 hour incubation in Marbrook flasks, cell cultures were harvested and 10×10^6 viable cells from each group were injected into (CBA x Balb) F_1 animals subjected to lethal irradiation. Appropriate CBA cell samples of culture flasks were independently used and subjected to the sheep red cell inhibition assay (Diener and Kraft, 1972). When injected into irradiated animals graft-versus-host death resulted within eight days.

This experiment was repeated many times and homologous (CBA anti Balb/c) antiserum was used in several added attempts. At no time was there evidence for tolerance induction *in vitro* using the purified H-2 antigen available to us. Although the transplantation antigen available was effective (i.e. 0.1 ml of 100 µg/ml sample added to CBA anti Balb/c antiserum HAT 1024 would reduce the titer fourfold), the purity of the protein was unknown. The heterogeneity of this

Group	Purified Balb/c H-2 Antigen *in Vitro*	Reciprocal Titers (HAT) of Heterologous Anti Balb/c Antiserum	Irradiated (950R) Animals Per Group (CBAxBalb/c)F_1
A	1 µg	100,000	5
B	"	10,000	5
C	"	100	5
D	"	10	5
E	"	1	5
F	"	10^{-1}	5
G	10 µg	--	5
H	1 µg	--	5
J	--	100	5
K	--	--	5

TABLE 3 - *In vitro* treatment of CBA spleen cells with transplantation antigen and antibody. 20×10^6 CBA spleen cells were exposed to microgram quantities of Balb/c transplantation antigen and serial dilutions of heterologous anti Balb/c antiserum. Following 72 hours incubation 10×10^6 harvested cells were injected into irradiated animals to determine graft-versus-host killer activity.

mixture may have precluded the antigen-antibody lattice formation necessary for tolerance induction *in vitro*.

C. Enhancement Studies

The successful enhancement studies with rat kidneys previously referred to prompted testing of the fetal heart in this regard. Accordingly, 18 day (CBAxBalb/c) F_1 hearts were transplanted into Balb/c adult recipients. Eight animals received 0.5 ml whole CBA anti Balb/c serum (HAT 1/1024) intravenously upon completion of grafting, and 0.5 ml intraperitoneally daily for the first four days after surgery. Graft survival was not significantly prolonged. Of six grafts which developed electrical activity five ceased to function on the eighth postoperative day and the remaining graft succumbed on the tenth day following surgery. This regimen was roughly comparable to that used by French and Batchelor (1969) in securing permanent renal survival in AS rats given an (August x AS) kidney. That the antiserum administered to these animals was of minimal toxicity was demonstrated in a further instance exploring the possibility of a peripheral enhancement effect. Eight fetal hearts were placed in a Falcon tube containing MEM/10% FCS to which sufficient homologous antiserum had been added to establish a HAT of 1/100. After eight hours incubation at 37°C all hearts were beating rhythmically. These were subsequently allografted, but underwent a normal rejection profile. The general validity of enhancement failure with this approach has recently been confirmed. Svehag and Schilling (1973) found significant prolongation of neonatal split cardiac allografts in the mouse, dependent upon recipient injection

with $F(ab)_2$ alloantibodies (thus devoid of cytotoxic potential) along with prednisone.

The failure of antigen-antibody mediated tolerance induction *in vitro* and *in vivo*, the current state of purification of transplantation antigens and the failure of enhancement studies prompted the approach to tolerance induction outlined in subsequent chapters. Although somewhat disheartening, initial failures at specific immunosuppression with the fetal heart graft model served to document the rather stringent requirements of the system.

CHAPTER VI

HEART ALLOGRAFT SURVIVAL AND VELOCITY SEDIMENTATION CELL SEPARATION

A. Introduction

Lymphoid cells have a limited life span and the immune system must be permanently replenished by bone marrow stem cells. This requires the continuous development of new immunocompetent cells as a result of somatic mutation, implying that self-reactive clones are generated as part of the process ensuring recognition of a large number of different antigens. The general lack of self reactivity is probably acquired by those lymphocytes which happen to carry recognition sites complementary to a self antigen. Although the actual mechanism of self tolerance is unknown, evidence suggests that it must occur before the relevant cells have reached complete immunocompetence.

This suggests that tolerance induction may be attempted following the rationale depicted in Figure 19. Lethally irradiated (950R) CBA mice will die within a few days from hematopoietic failure if they are not reconstituted with syngeneic bone marrow. Consider two groups of mice transplanted with Balb/c fetal hearts just following irradiation at a time when they are immunologically incompetent. If the first group of mice are reconstituted with whole bone marrow, which includes both stem cells and immunocompetent cells and the second group receive stem cells alone, the fate of the allografts in each case will,

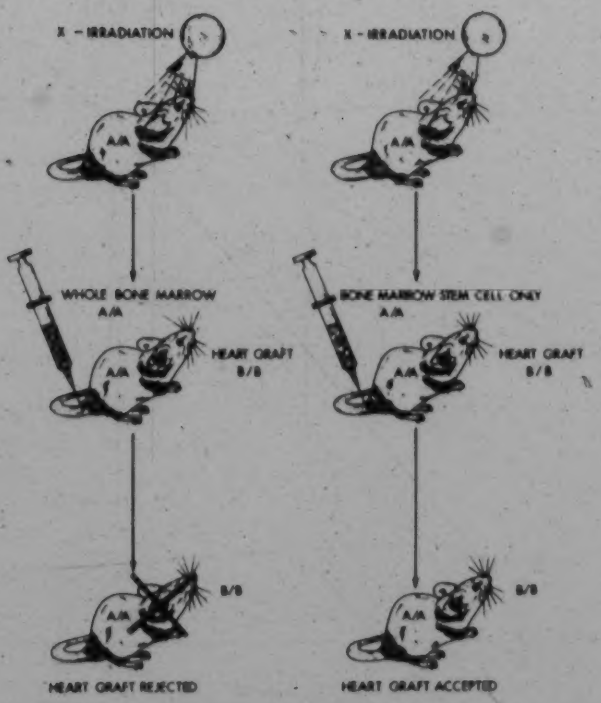


Figure 19 Repopulation of an irradiated heart allografted animal with syngeneic bone marrow eventuates in allograft rejection. Repopulation with purified hematopoietic stem cells should induce tolerance in stem cell progeny with cardiac allograft survival.

theoretically, differ. CBA graft recipients given syngeneic whole marrow should reject a Balb/c heart due to the presence of immunocompetent lymphocytes in the reconstituting marrow. However, Balb/c hearts placed in CBA recipients injected with hematopoietic stem cells alone should survive indefinitely, becoming permanently accepted as self as proliferation and differentiation of stem cells in the presence of antigen determines tolerance.

The problem revolves then, around the isolation of the primordial stem cell and excluding it from immunocompetent cell progeny. Quite apparently this approach to experimental tolerance induction is relevant to the clinical and laboratory problems seen in graft-versus-host disease. In allografting whole marrow or other lymphoid tissues the immunocompetent cell inoculum recognizes foreign antigens and mounts an immune response. Specific deletion of immunocompetent cells has for this reason alone become a promising experimental development.

Pharmacologic agents may be useful in this regard. Immunocompetent cells can be stimulated by nonspecific mitogens such as phytohemagglutinin (Nowell, 1960), concanavalin A (Knight and Thorbecke, 1971) or by specific transplantation antigens (Reisfeld and Kahan, 1971; Viza, Degani *et al*, 1968). Consequent proliferation of cells renders them susceptible to pharmacologic attack by agents which interfere with normal nucleic acid synthesis and results in the specific elimination of responsive cells.* In recent experiments both radioactive tritiated thymidine (Salmon, Krakauer *et al*, 1971) and 5-bromodeoxyuridine (BuDR;

*The author's experiments in this regard are documented in Appendix Section II.

Zoschke and Bach, 1971) have been used to selectively remove stimulated (immunocompetent) lymphocytes *in vitro*. Cell cultures treated with cytotoxic agents active during cell division will, at an appropriate dosage level, retain stem cell activity. This is so because stem cells divide rather slowly and remain virtually unaffected (Bruce, Meeker *et al*, 1966; Lajtha, 1967).

More extensive, however, have been experiments designed to purify bone marrow stem cells by physical means. These have been based on differences between lymphoid cells with respect to size (Miller and Phillips, 1969) and density (Turner, Siminovitch *et al*, 1967; Worton, McCulloch *et al*, 1969). The experiments of Phillips and Miller (1970) demonstrated that cells in mouse and bone marrow and spleen which were capable of graft-versus-host activity could be defined as a population of small, slowly sedimenting cells which fell through a fetal calf serum gradient with a rate of modal distribution of about 3 mm/hr. Stem cells, or cells defined by their ability to form hematopoietic colonies in the spleens of irradiated mice (Till and McCulloch, 1961) tended to be larger in size and sedimented more quickly (Figure 20). Although clinical trials with bone marrow subjected to separation procedures (Speck, Pooren *et al*, 1971) have enjoyed only limited success, it was decided to test whether stem cells could be isolated from immunocompetent cells. The assay system (graft-versus-host activity) of Phillips and Miller (1970) permitted only short term assessment of cell reactivity; reconstitution of the irradiated allografted mouse would permit long-term evaluation of the effect of cell separation. Stem cell purification would, apparently, permit reconstitution of a lethally irradiated mouse with acceptance as "self" of an allogeneic fetal heart.

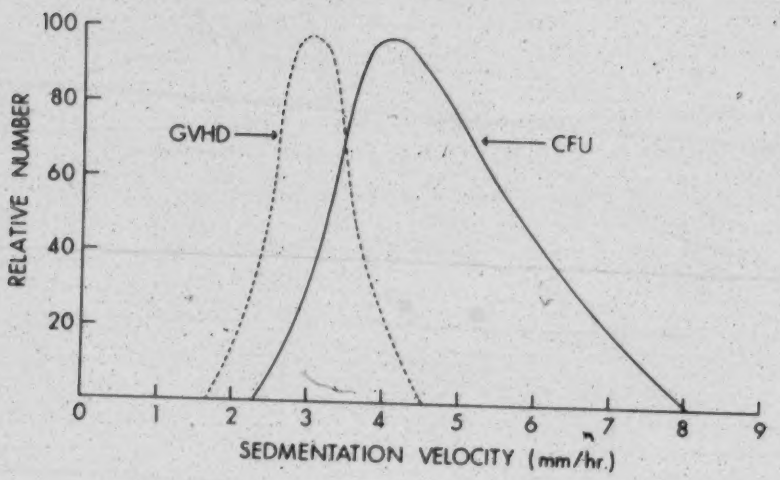


Figure 20 Velocity sedimentation cell separation of mouse bone marrow and spleen: the modal distribution of immunocompetent (GVHD) and stem (CFU) cells. Purified stem cells sediment most rapidly and may be separable from immunocompetent cell fractions.

[Adapted from Miller and Phillips, 1970.]

Accordingly, CBA mouse marrow and spleen were subjected to cell separation on the basis of velocity sedimentation. Fractionated hematopoietic tissue was then used to reconstitute syngeneic irradiated and heart allografted mice.

B. Methods

1. Cell Separation

The technique of velocity sedimentation cell separation has been described. After sedimenting the cells for 2.5 hours at 4°C, 15 ml fractions were collected. Fractions were pooled on the basis of calculated velocity of sedimentation values as illustrated in Figure 21. This separation has been independently applied to the study of immunocompetent cells by Diener, Kraft *et al* (1973) and found to give similar values to those described by Miller and Phillips (1970).

2. Reconstitution of Irradiated Animals

Mice were reconstituted one day after irradiation (950R) with an intravenous injection of lymphoid cells. Reconstituting inocula of lymphoid cells consisted of (a) unfractionated bone marrow, (b) unfractionated bone marrow plus spleen cells, (c) unfractionated marrow plus fractionated spleen cells, and (d) fractionated spleen cells.

3. Heart Allograft Insertion and Subsequent Evaluation

On the day of irradiation CBA mice were transplanted with a Balb/c fetal cardiac graft to the right ear as described in Chapter II. In initial experiments loss of fetal heart activity in irradiated repopulated mice did not occur in the first several weeks following graft insertion. For this reason, and to permit animals to recover

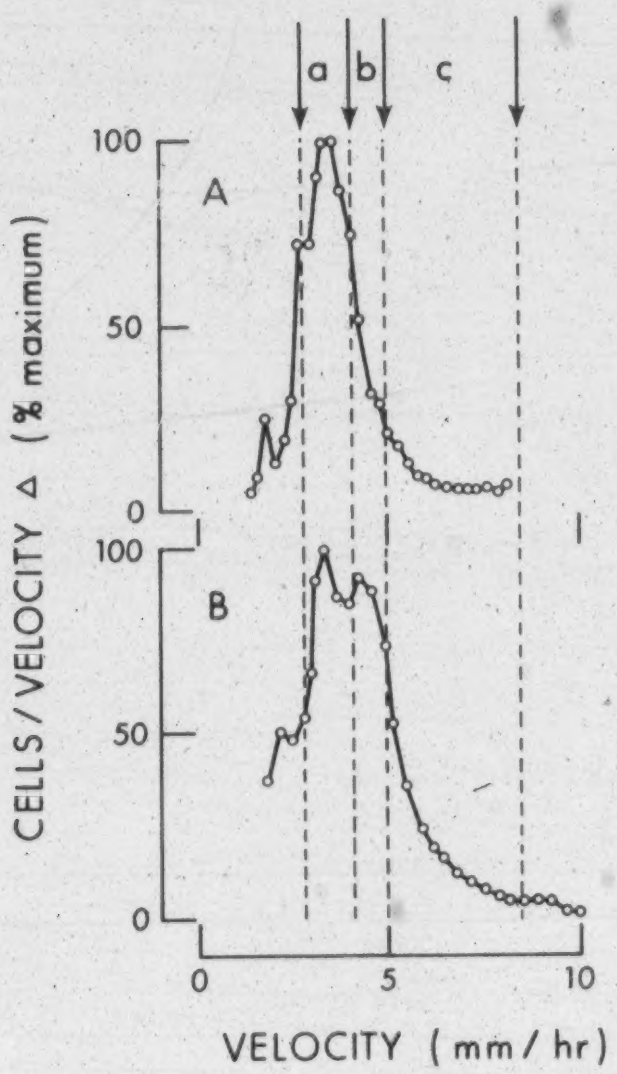


Figure 21 A typical velocity sedimentation distribution profile of:

A - Normal spleen nucleated cells. A pooled sample of 300 million total cells, from two mice, was applied in 30 ml 3% FCS PBS to the staput.

B - Normal bone marrow non-erythroid cells. A pooled sample of 90 million cells from three mice was applied in 15 ml 3% FCS PBS.

The dotted line encompasses the velocity ranges used for pools of: a - small, b - medium, c - large cells.

Fraction a contains the majority of immunocompetent cells; fractions b and c are stem cell rich.

from irradiation, electrocardiography was first performed two weeks after allotransplantation. Those animals in which grafts had "taken" and exhibited electrical activity were then followed by serial electrocardiography at one week intervals.

C. Results

1. Whole Marrow and Spleen

When irradiated CBA mice were reconstituted with 0.1×10^6 syngeneic bone marrow cells, the mean rejection time was four weeks (Figure 22A). Since rejection was presumably due to the presence of mature immunocompetent cells (ICC) in the inoculum, similar experiments were then performed, adding 2×10^6 (or a twentyfold increase) of syngeneic spleen cells to the marrow inoculum. The addition of spleen cells, known to contain a larger proportion of ICC than bone marrow did not, however, increase the rate of allograft rejection (Figure 22A). In fact, in further experiments, repopulation of animals with 10×10^6, 20×10^6 and 50×10^6 spleen cells did not appear to affect the ability of allografts to survive in the four week interval following transplantation. Cell separation experiments were thus indicated to probe the specific effects of ICC and stem cell enriched fractions in the rejection process.

2. Fractionated Marrow and Spleen

Reconstitution of animals with 0.1×10^6 ICC enriched bone marrow (Figure 22B) was no more effective in rejecting the allograft heart than other fractions presumably devoid of these cells. This suggested that cells other than mature ICC were involved in graft

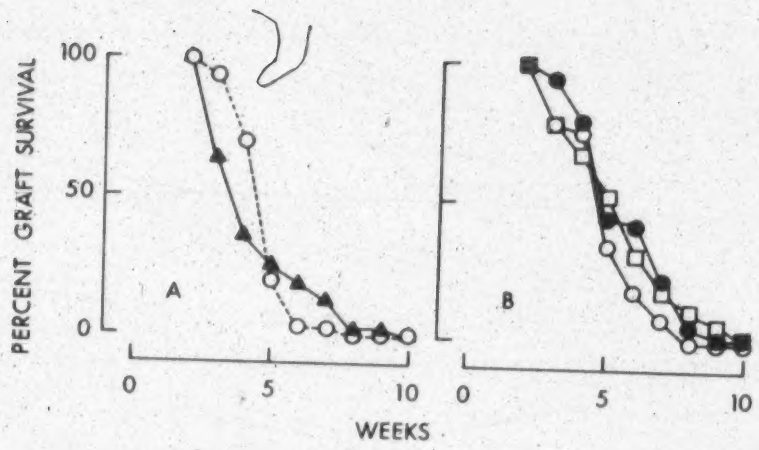


Figure 22 Cardiac transplant survival in mice lethally irradiated, allografted and reconstituted with hematopoietic tissue:

A ▲-Unfractionated bone marrow (0.1×10^6 cells; 33 mice)

○-Unfractionated bone marrow plus spleen (0.1×10^6 bone marrow cells; 2×10^6 spleen cells; 15 mice)

B □-Fractionated bone marrow small cells (0.1×10^6 cells; 27 mice)

○-Fractionated bone marrow, medium cells (0.1×10^6 cells; 20 mice)

●-Fractionated bone marrow, large cells (0.1×10^6 cells; 36 mice)

rejection in this system, one possibility being that ICC precursors were present in the bone marrow inoculum in the stem cell rich sedimentation fraction. When stem cell rich fractions of spleen, either alone or with bone marrow, were used to repopulate allografted mice the results were quite different (Figure 23A&B). Grafted animals which received only the large or rapidly sedimenting spleen cells maintained their grafts longer than recipients of bone marrow alone. In addition, this high velocity fraction gave evidence of inhibiting the rejection process since three animals carried functional allografts longer than four months following reconstitution with both large spleen cells and bone marrow. Similarly, reconstitution of irradiated animals without syngeneic bone marrow but with large spleen cells alone permitted four of 17 heart grafted animals to retain allograft function up to 12 weeks post irradiation. Since mice which had successfully maintained an allograft heart for 12 weeks post irradiation and reconstitution did not subsequently reject the allograft, they were considered, in effect, tolerant of their allografts. Such animals are referred to, in subsequent experiments, as tolerant mice.

D. Discussion

The initial finding that syngeneic bone marrow cells in very small numbers used to reconstitute a CBA mouse subjected to irradiation and cardiac allografting eventually lead to rejection allows several possibilities. The cells which ultimately caused rejection may have been mature ICC, present in small numbers in the bone marrow inoculum, which required 4-5 weeks for sufficient clonal expansion to reject the

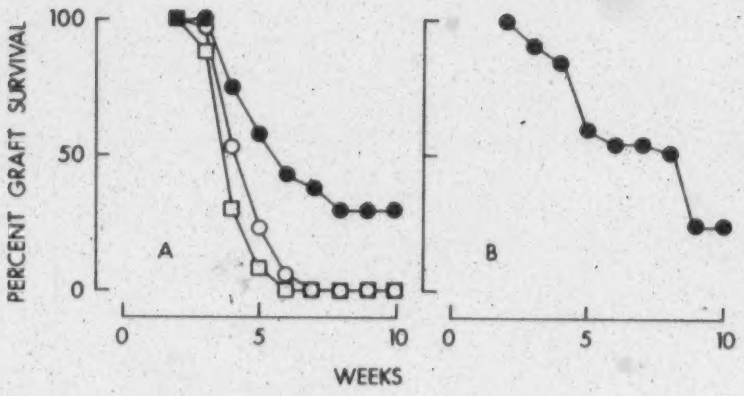


Figure 23 Cardiac transplant survival in mice lethally irradiated, allografted and reconstituted with hematopoietic tissue:

A -- Unfractionated bone marrow 0.1×10^6 cells plus

□-2×10^6 small spleen cells (17 mice)

○-2×10^6 medium sized spleen cells (14 mice)

●-2×10^6 large spleen cells (10 mice)

B ●-Large spleen cells alone (2×10^6 cells; 17 mice)

the graft. Alternatively, the majority of ICC, present at the time of rejection, may have been differentiation products of precursor cells in bone marrow. Since the addition of 2×10^6 spleen cells to the reconstituting inoculum did not accelerate graft rejection, the small numbers of ICC in bone marrow at the time of reconstitution were apparently not a limiting factor. Indeed, velocity sedimentation of bone marrow showed that the ICC enriched fractions of marrow were no more efficient at rejecting the graft than the faster sedimenting, stem cell rich fractions. The situation with spleen cell reconstitution was different. Irradiated mice which received only the large or rapidly sedimenting spleen cells (without bone marrow) showed allograft survival significantly longer than in recipients of whole or fractionated marrow. In addition, the high velocity spleen cell fraction gave evidence of inhibiting the rejection process, since three of ten animals carried functional allografts longer than four months following reconstitution with both large spleen cells and whole bone marrow. In contrast then, bone marrow cells permitting allograft rejection were spread over the entire range of velocity sedimentation values investigated, but spleen cells differed, the large cell fraction thereof prolonging allograft function with permanent graft survival (>12 weeks) in 25-30% (7/27) of animals. Immunocompetent precursor cells may thus be present in the high velocity sedimentation fraction of bone marrow and absent in spleen or, alternatively, a class of large spleen cells which is absent in bone marrow may be inhibitory, promoting graft survival.

CHAPTER VII

IMMUNOSUPPRESSION AND ALLOGRAFT SURVIVAL

A. Introduction

In the previous chapter it was shown that cardiac allograft rejection in an irradiated animal repopulated with syngeneic marrow occurs within four to five weeks. Apparently within this time interval sufficient immunocompetent cells are generated to destroy the allograft heart. Since prolonged graft survival has previously been noted in renal allografted rats treated with (1) homologous antiserum (French and Batchelor, 1969; Stuart, Saitoh *et al*, 1968), (2) short term anti-lymphocytic serum (Guttmann, Lindquist *et al*, 1969), these modalities could, conceivably, produce long standing graft survival in the irradiated mouse model. It is to be noted that long standing graft survival with either ALS or homologous antisera has been routinely documented only with F_1 to parent renal allografts in rats and, occasionally, with canine kidneys transplanted across an indeterminate histocompatibility barrier (Shanfield, Wolf *et al*, 1973).

The possible influences of homologous graft directed antisera have been described previously. Antilymphocyte globulin preparations are less specific in their effects but have demonstrated a variety of immunosuppressive activities. They may destroy, most particularly, the circulating lymphocytes (Levey and Medawar, 1966; James, 1967), render lymphocytes immunologically inactive (Boak, Fox *et al*, 1967) or

coat lymphocytes, effecting their subsequent removal from the circulation (Martin and Miller, 1967).

The purpose of the investigations described in this chapter was to investigate whether or not different antisera, given in short term discontinuous fashion could deter the normal rejection response and produce long term cardiac allograft survival. The model system again employed CBA mice which were irradiated (950R), cardiac allografted and reconstituted with 0.1×10^6 syngeneic bone marrow cells.

B. The Effect of Different Antisera

1. Horse Anti Mouse Thymocyte Serum and Globulin

During irradiation recovery mice were injected with 0.05 ml HAMTS intraperitoneally (IP) at weeks 2, 3, 4 and 5. The dosage of HAMTS (Hemagglutinin titer 500,000) was selected empirically as the minimal amount which would permit survival of the irradiated mouse. The stem cell toxicity of such preparations is well documented (Gallagher, Richie *et al*, 1972) and may, in fact, be removed by absorption with spleen cells (Trentin and Judd, 1973). The experience with a small group of mice treated with HAMTS (Figure 24), demonstrated, however, that long term survival could be obtained with whole serum. A larger group of 45 mice were treated with the less toxic IgG (HAT 500,000) preparation administered IP 2 ml once weekly either at weeks 2 and 3, or weeks 2-5. Much the same immunosuppression was obtained in this group of animals, with 17% of animals bearing grafts beyond the twelve week period. The immunosuppression obtained with HAMTS IgG 8 weeks

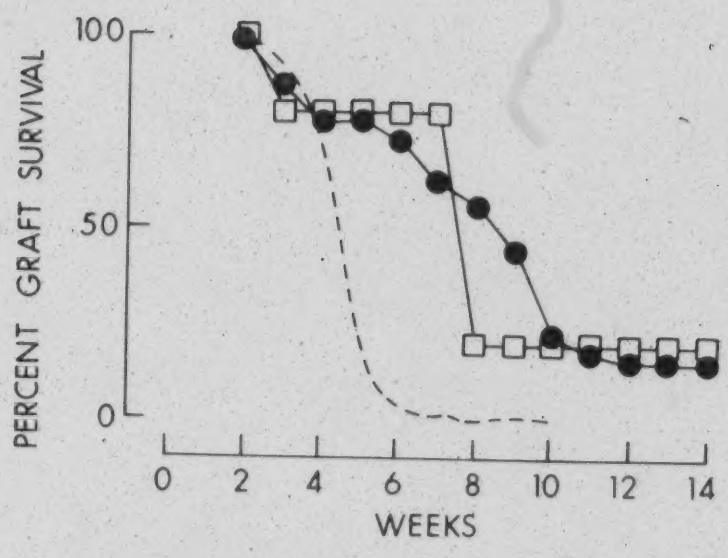


Figure 24 Effect of horse anti mouse thymocyte serum (HAMTS) on allograft survival. CBA adult mice were irradiated (950R), allografted with a Balb/c fetal heart and reconstituted with 0.1×10^6 syngeneic bone marrow cells.

□ 0.05 ml HAMTS intraperitoneal injection weeks 2, 3, 4, 5 post irradiation; 5 mice

● 0.2 ml HAMTS IgG one intraperitoneal injection weeks 2 and 3 or weeks 2, 3, 4, 5 post irradiation; 45 mice

--- normal pattern of rejection without treatment; 33 mice

At 8 weeks post irradiation the immunosuppression obtained with HAMTS IgG treatment is very significant ($p<0.001$, x^2 test)

post irradiation was very significant ($p<0.001$) compared with non-treated control animals.

2. Rabbit Anti Balb/c IgG

Antiserum prepared in rabbits in response to injections of Balb/c mastocytoma cells was used to obtain a rabbit anti Balb/c IgG (RAB IgG) preparation. In an initial experiment three animals treated with 2.0 ml of the IgG preparation (IP injection weeks 2-5) retained indefinite allograft activity. Further preparations were not as effective, however. The graph depicted in Figure 25 is a summary of seven subsequent experiments depicting the loss of allograft function in 84 animals demonstrating initial cardiac activity. The hemagglutinating activity of these preparations was comparable to that of the HAMTS IgG but a larger volume (2.0 ml) was administered on weeks 2, 3 or 2, 3, 4 and 5. The activity of RAB IgG was much less potent than HAMTS IgG, and only two grafted animals retained allograft activity beyond twelve weeks post irradiation.

3. Effect of CBA Anti Balb/c Serum, IgG and IgM

The intraperitoneal injection of 1 ml of whole CBA anti Balb/c serum (HAT 1512) at weeks 2 and 3 post irradiation significantly prevented rejection in 18 animals, but only during the period of administration (Figure 26). Administration of 0.5 ml of the IgG fraction of this serum at weeks 2, 3, 4, 5 was not markedly effective, but did produce one long-term graft bearing animal. Administration of the IgM fraction in the same manner was ineffective.

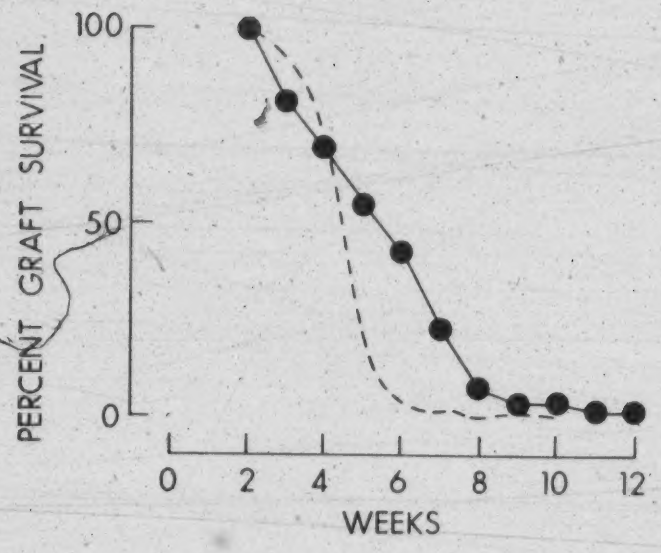


Figure 25 Effect of rabbit anti Balb/c (RAB) IgG on allograft survival. CBA adult mice were irradiated (950R), allografted with a Balb/c fetal heart and reconstituted with 0.1×10^6 syngeneic bone marrow cells.

● 2.0 ml RAB IgG intraperitoneal injection weeks 2, 3 or 2, 3, 4, 5 post-irradiation; 84 animals

--- normal pattern of rejection without treatment; 33 animals

At 8 weeks post-irradiation the immunosuppression obtained with RAB IgG treatment is not significant (x^2 test)

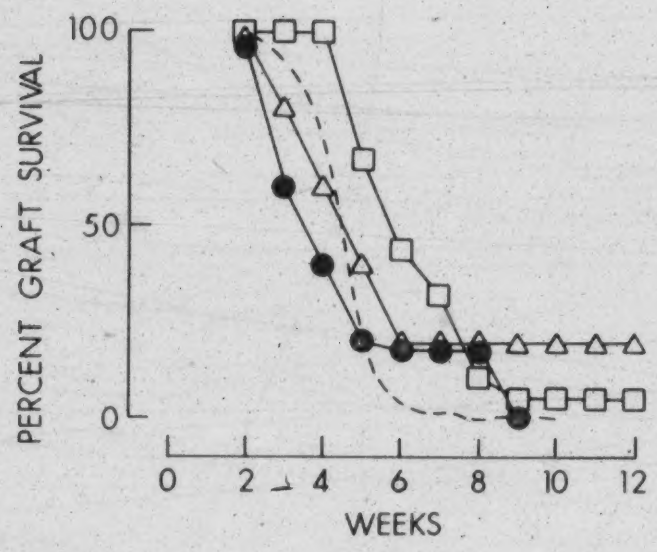


Figure 26 Effect of CBA anti Balb/c serum, IgG and IgM on allograft survival. CBA adult mice were irradiated (950R), allografted with a Balb/c fetal heart and reconstituted with 0.1×10^6 syngeneic bone marrow cells.

□ 1.0 ml CBA anti Balb/c serum intraperitoneal injection weeks 2, 3 post-irradiation; 18 animals

△ 0.5 ml CBA anti Balb/c IgG intraperitoneal injection weeks 2, 3, 4, 5; 5 animals

● 0.5 ml CBA anti Balb/C IgM intraperitoneal injection weeks 2, 3, 4, 5; 5 animals

--- normal pattern of rejection without treatment; 33 animals

At 4 to 6 weeks post irradiation the immunosuppression obtained with CBA anti Balb/c serum treatment is significant. ($p<0.01$; x^2 test)

C. Repopulation of Thymectomized Animals

Ten CBA mice were adult thymectomized (Miller, 1961) one month prior to irradiation (950R), cardiac allografting and repopulation with syngeneic whole bone marrow. As expected, this proved to be markedly immunosuppressive and permitted 60% of animals to bear permanent cardiac allografts (Figure 27). This confirms the established role of the thymus in maintaining cell mediated immunity. The fact that four of the ten animals did reject their allografts may suggest either (1) thymic regeneration occurred in these animals (incomplete thymectomy), or (2) that there were sufficient graft reactive cells present in the bone marrow inoculum to eventually expand sufficiently to effect graft rejection. For the purposes of this study, however, graft survival in thymectomized animals is insufficient since adult thymectomy produces non-specific cellular immune deficiency (Miller and Osoba, 1967).

D. Immune Status of Animals Treated with Antisera

1. Repeat Allografts

The most effective means of producing graft tolerant mice was by administration of HAMTS. In order to demonstrate that immune recovery had occurred after this treatment, a total of 16 animals were selected after they had shown no graft electrical activity for three successive weeks. These animals were then given a second Balb/c heart graft in the opposite ear. In none of these animals was graft electrical activity apparent beyond one week following the second graft insertion. In other words, in animals, which despite HAMTS treatment,

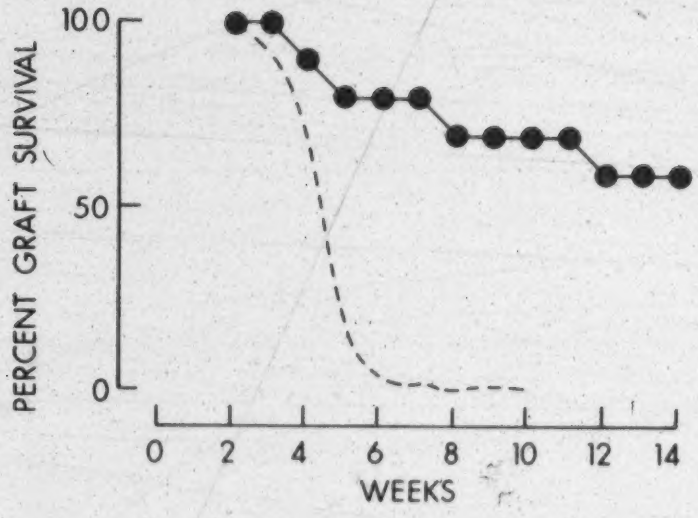


Figure 27 Effect of thymectomy prior to reconstitution. CBA adult mice were thymectomized. One month later they were irradiated, allografted with a Balb/c fetal heart and reconstituted with 0.1×10^6 syngeneic bone marrow cells.

● CBA mice treated with prior thymectomy; 10 animals

--- normal pattern of rejection without thymectomy; 33 animals

Beyond 4 weeks post-irradiation the influence of prior thymectomy is significant ($p<0.01$; x^2 test)

rejected their grafts, immunologic recovery was evident such that a second allograft did not show prolonged survival.

2. Immunocompetence of Tolerant Mice

As with tolerance induction by velocity sedimentation techniques, mice which had permitted allografts to survive longer than 12 weeks beyond initial irradiation and subsequent antiserum treatment were considered operationally tolerant. The immunocompetence of such mice was tested *in vitro*. Spleen cells from tolerant mice could react normally in tissue culture, as outlined in the following chapter. Tolerant mice were thus immunocompetent. Tolerant animals were effectively given a second Balb/c heart graft by the studies described in Chapter IX. Lymph node cells from tolerant mice transferred tolerance to secondary irradiated recipients reconstituted with whole bone marrow. Several tolerant animals given a "third party" (C57Bl) graft were capable of rejecting this graft while maintaining Balb/c allograft activity.

E. Conclusions

Long-term cardiac allograft survival can be obtained by administration of either heterologous or homologous antisera. In those treated animals which reject their graft, subsequent repeat allografts result in early rejection (within 7 days) indicating the return of immunologic competence in these animals. Those animals which retained their grafts longer than 12 weeks post-irradiation were fully reactive *in vitro* and thus immunocompetent. In these studies the

injection schedules, sources of antisera and routes of administration were, of necessity, empirically selected. Certainly investigation of these factors would improve the number of animals specifically tolerant of allograft hearts. Short term discontinuous treatment of irradiated mice with heterologous antisera did not prevent the subsequent development of immunocompetence.

CHAPTER VIII

RECOGNITION OF ALLOANTIGENS IN TOLERANT MICE

A. Introduction

Early researchers in the field of transplantation tolerance (Billingham, Brent *et al*, 1956) formed the opinion that tolerance was due to a central failure of the immune response rather than to interference with a normally reactive immune system. This dogma has persisted without challenge until the last several years. It has recently been proposed that tolerance is in reality due to the interposition of some protective serum factor between an aggressive lymphoid cell and the target it would otherwise act upon. For example the Hellströms (1970a), Voisin, Kinsky *et al* (1968) and Ceppelini (1971) and their associates have suggested that some types of tolerance may be effected by antibodies which somehow suppress antigenic recognition by immunologically competent cells.

This implies that immunologically competent cells exist in tolerant animals and that removal from a tolerance sustaining milieu would demonstrate immune responsiveness. There are a number of relevant studies in this regard. Mintz and Silvers (1967) have shown that allophenic mice, chimeric since the eight cell stage because of whole embryo fusion, are operationally tolerant to themselves and to both parental strains. Nevertheless, they possess both immune cells capable

of specifically destroying parental strain fibroblasts *in vitro* and specific serum blocking factors capable of preventing that destruction (Wegmann, Hellström *et al*, 1971). On the other hand, studies by Atkins and Ford (1972) in rats rendered tolerant to transplantation antigens by the neonatal injection of F_1 hybrid marrow, have shown that tolerant thoracic duct lymphocytes (TDL) do not effect the graft-versus-host potential of normal TDL and are presumably nonreactive. Similar studies by Bildsoe and Ford (1971) have established that splenic lymphocytes from rats rendered tolerant by marrow cell infusion at birth were inactive in the popliteal lymph node assay for graft-versus-host disease.

The situation in animals rendered tolerant to allografts (defined as permanent graft survival without continuing immunosuppression) is equally unclear. Hellström, Hellström *et al* (1970c) have demonstrated that canine irradiation chimeras possess lymphoid cells which kill host fibroblasts *in vitro* and that serum can specifically abrogate this cytotoxic effect. On the other hand Grant, Leuchars *et al* (1972) have studied murine irradiation chimeras. Spleen cells removed from tolerant animals were unable to kill tumor cells of host antigenicity *in vitro* and that further, serum taken from these chimeras did not prevent immune cells of donor genotype being cytotoxic to host target cells. For animals bearing permanent grafts following treatment with homologous antisera, the situation is somewhat more consistent. Stuart, Fitch *et al* (1971) and French, Batchelor *et al* (1971) both demonstrated that rats bearing permanent renal allografts following short term antiserum treatment were capable of mounting a cell mediated response to graft antigens. Indeed, the earlier studies

of Voisin, Kinsky *et al* (1968) had shown that highly tolerant animals do, in fact, develop immunological reactions against the transplantation antigens of the tolerated lines and that, further, the appearance of significant levels of anti H-2 antibodies in the tolerant mouse did not signal the rejection of the graft. Sera from highly tolerant animals were found to contain specific enhancing antibodies able to passively transfer to normal mice the ability to tolerate a graft from the donor strain.

The purpose of the investigations described in this chapter was to determine the specific responsiveness of adult CBA mice operationally tolerant to Balb/c cardiac allografts. Since tolerant animals were obtained by a variety of means following irradiation, (including syngeneic stem cell reconstitution, which is perhaps analogous to the induction of self tolerance during ontogeny), the system invited investigation in this regard.

B. Methods

The basic plan of the experiment was to compare the capacity of spleen cells from normal and from tolerant mice to become sensitized *in vitro* to Balb/c mastocytoma cells and subsequently effect ^{51}Cr release from labelled mastocytoma cells.

CBA spleen cells, prepared as previously described, were obtained from normal and tolerant mice. 20×10^6 spleen cells were cultured *in vitro* in the presence of 1×10^4 Balb/c mastocytoma cells in MEM/FCS. After 96 hours incubation cells were harvested and washed with L/FCS (450G×7.5 min) and were reconstituted with 10 ml L/FCS.

Spleen cell counts were obtained and after further centrifugation (450G×7.5 min) the cells were suspended in MEM/FCS. Control cultures consisted of spleen cells derived from normal CBA and Balb/c mice with and without addition of mastocytoma cells during the incubation period. Radiolabelled (^{51}Cr) Balb/c mastocytoma cells were prepared as described in Chapter II. Reaction mixtures (0.5 ml MEM/FCS) containing 1×10^4 labelled mastocytoma cells were incubated with 1×10^6 cells derived from tissue culture. In further control experiments introduced at this stage, the Brunner assay was carried out with fresh (non-cultured) CBA and Balb/c spleen cells, as well as with test cultured cells. All experiments were carried out in triplicate or quadruplicate reaction mixtures. After nine hours incubation, the previously described calculations determined (1) percent supernatant release, (2) supernatant total release, and (3) converted total release of ^{51}Cr.

C. Results

The data obtained from a single representative experiment are shown in Table 4. Since incubation of spleen cells in tissue culture was done with several flasks per test procedure, and the reaction mixtures derived from single culture flasks were read in triplicate samples, supernatant and precipitate counts represent mean figures with standard deviations. Converted total release (CTR) and mean CTR figures (from triplicate procedures) are finally computed. Mean CTR percentages compare the ^{51}Cr release of control or nonreactive mixtures (usually normal Balb/c or CBA cells in tissue culture without mastocytoma cells) with the reactivity of CBA spleen cells cultured in the presence of Balb/c

	Supernatant Counts	Precipitate Counts	SR (%)*	STR (%)+	CTR (%)¶	Mean CTR of 3 Exper (%)
Normal CBA spleen + mastocytoma cells *in vitro* (96 hrs)	5586±107	5902±58	59.9	69.2	58.3	59.0
Normal CBA spleen *in vitro* (96 hrs)	2700±25	9061±54	2.7	28.5	3.3	5.9
Normal Balb/c spleen *in vitro* (96 hrs)	2665±56	9538±195	0	26.1	0	0
CBA spleen (fresh)	3319±86	8729±314	15.0	35.0	12.1	12.1
Balb/c spleen (fresh)	3471±101	8627±167	17.2	36.4	13.9	13.9
Tolerant CBA spleen + mastocytoma cells *in vitro* (96 hrs)	5145±110	6627±383	51.2	64.6	52.1	41.5

*SR (%) = Supernatant Release (%)
+STR (%) = Supernatant Total Release (%)
¶CTR (%) = Converted Total Release (%)

TABLE 4 - Comparison of the ability of spleen cells from normal and Balb/c heart graft tolerant mice to effect ^{51}Cr release from labelled Balb/c mastocytoma cells. Normal and tolerant spleen cells were cultured *in vitro* with Balb/c mastocytoma cells for 96 hours. Subsequently harvested spleen cells were tested for their effect on radiolabelled tumor cells, as assayed by ^{51}Cr release over a nine hour incubation period.

mastocytoma cells for four days. As shown in this table, normal CBA spleen cells, sensitized to Balb/c alloantigens *in vitro*, are able to subsequently effect ^{51}Cr release (i.e. exhibit killer cell activity) from radiolabelled Balb/c mastocytoma cells. However spleen cells derived from a CBA mouse tolerant to a Balb/c cardiac allograft, subjected to the same mastocytoma exposure, also effect release of label from target tumor cells.

Data from eleven such experiments are summarized in Table 5. Spleen cells from both normal and Balb/c heart graft tolerant mice, upon exposure to Balb/c alloantigens *in vitro*, were able to subsequently express killer activity against Balb/c tumor cells. In fact, there was no statistically significant difference between the cell mediated immunity demonstrated by spleen cells of either normal or tolerant mice. In contrast, however, exposure of spleen cells to mastocytoma alloantigens *in vitro* rendered them capable of major killer activity, significantly different, in all cases, from (1) CBA spleen cells cultured without tumor, (2) CBA spleen cells freshly prepared from a normal mouse, (3) Balb/c cells cultured in the presence of tumor cells, or (4) Balb/c cells freshly prepared from a normal donor.

D. Discussion and Conclusions

CBA mice rendered tolerant to Balb/c fetal cardiac allografts by a variety of methods did not exhibit diminished or absent immune reactivity to the alloantigens of the graft. It follows that the persistence of graft function may be due to either a change in graft immunogenicity (graft adaptation), or to cellular or humoral mechanisms

Expt	Normal CBA Spleen + Mast *in Vitro* (% release)	Normal CBA Spleen Tissue Culture (% release)	Normal Balb/c Spleen Tissue Culture (% release)	CBA Spleen (Fresh) (% release)	Balb/c Spleen (Fresh) (% release)	Tolerant CBA Spleen + Mast *in Vitro* (% release)
1	59.0	5.9	0	12.1	13.9	41.5
2	16.3	-2.9	0	9.4	7.0	11.5
3	68.6	15.4	0	1.1	1.2	70.4
4	42.8	10.8	0	3.8	-	42.2
5	53.0	0	-3.8	-0.2	0.1	30.5
6	10.5	0	12.9	13.5	8.1	16.5
7	41.5	0	11.0	5.2	4.3	59.0
8	9.0	19.0	0	-	-	11.5
9	10.6	0	-	-	-	14.9
10	36.4	0	-	-	-	36.1
11	3.7	-6.9	0	1.0	-2.8	14.2

Students' Paired T Test (1) Normal CBA + *in vitro* mast versus tolerant CBA + *in vitro* mast; $p>0.5$
(2) Normal CBA + *in vitro* mast versus normal CBA tissue culture; $p<0.005$
(3) Normal CBA + *in vitro* mast versus fresh CBA; $p<0.01$

TABLE 5 - Comparison of spleen cell activity from tolerant and normal mice *in vitro*. Figures listed represent the mean converted total release of ^{51}Cr from labelled Balb/c mastocytoma cells effected by spleen cells from normal and tolerant CBA mice. In 11 individual experiments spleen cells were cultured in the presence of mastocytoma cells for 96 hours. Harvested spleen cells were tested for killer activity (^{51}Cr release) on radiolabelled tumor cells.

present in the tolerant animal, preventing graft destruction. That graft adaptation is probably of little significance has been suggested by the studies of Stuart, Fitch *et al* (1971) and Warden, Reemtsma *et al* (1973). Allografts which enjoy permanent survival following homologous antiserum treatment in the rat are normally rejected upon transplantation into a secondary untreated recipient.

This work agrees then, with the findings of the Hellströms and colleagues, referred to earlier. Criticism of experiments in which similar immune reactivity has been demonstrated in animals bearing permanent functional grafts may, however, be possible. One might suggest, for instance, that immune reactivity towards graft antigens in such animals, though demonstrable at a time long past that expected for graft rejection, eventually wanes and disappears. In other words, for permanent graft acceptance, classical enhancement phenomena may be a stage preceding the development of central tolerance. There is certain evidence suggestive in this regard. Thoenes and White (1973) studied rat renal allograft survivors established by initial postoperative treatment with homologous antiserum. During the long course of allograft recipient survival, there was a slow decline in demonstrable antigraft antibodies. Subsequent to this, skin allografts (histocompatible with the initial renal allograft) were accepted by the animal, suggesting that a state of auto-enhancement is followed by specific non-reactivity. Similarly Marquet and van Bekkum (1973), studying enhanced heterotopic heart allografts in rats, found that the antidonor activity of peripheral blood lymphocytes was inconstant, depending on the time of graft survival. Unmodified or increased lymphocyte reactivity was

followed by a loss of reactivity, suggesting that, at least for the rat strains involved, graft survival is temporarily associated with a central failure of the immune response. Following this train of thought then, reduced alloantigen reactivity in heart graft tolerant mice, could be apparent at a later stage of survival.

CHAPTER IX

TOLERANCE TRANSFER WITH LYMPH NODE CELLS

A. Introduction

The normal reactivity of splenic lymphocytes in heart graft tolerant animals suggested that either cellular or humoral immune mechanisms were thwarting an expected rejection response. There are, in fact, several studies which suggest that tolerant lymphocytes or products of tolerant lymphocytes may interfere with an expected immune response. As mentioned earlier, Nisbet (1971) has produced tolerant mice by an eight week period of parabiotic union. Spleen cells from adult CBA mice (carrying the T6 marker chromosome) rendered tolerant to (CBAxC3H)F_1 hybrid mice by long-term parabiosis were injected into newborn syngeneic animals. This injection rendered the newborn CBA mice tolerant to a subsequent (CBAxC3H)F_1 skin graft, placed in the neonatal period. A small fraction of noninjected animals reacted similarly, however, and retained F_1 skin grafts, apparently due to the weak antigenic differences involved and, perhaps, recipient immunologic immaturity. The studies of McCullagh (1970;1972) are, once more, encouraging. Rat hosts tolerant to sheep erythrocytes render normal syngeneic lymphocytes specifically tolerant. Tolerance to sheep erythrocytes could be effected by parabiosis of tolerant and normal rats or by the transfer of cell suspensions from a tolerant donor to a normal recipient. Thoracic duct lymphocytes and spleen cells from

tolerant donors were highly effective in transferring tolerance, but bone marrow cells were completely lacking in this capacity. McCullagh hypothesized that a population of cells is tolerant because it contains tolerant cells in which a particular reactivity has been repressed; so called repressor cells.

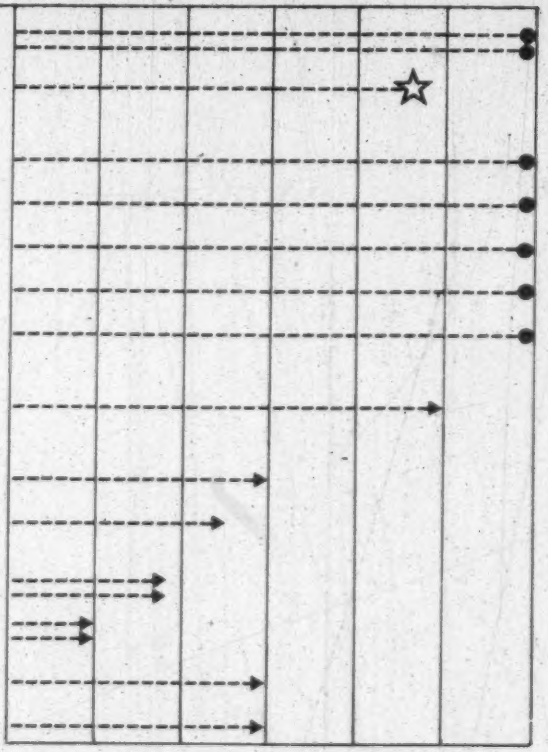
If immunological tolerance is an active state of repression *in vivo*, it would be predicted that tolerant lymphocytes might transfer this effect on exposure to (1) the tolerogen (i.e. the heart graft) and (2) sufficiently small numbers of relevant immunocompetent cells (i.e. the irradiated mouse repopulated with syngeneic bone marrow).

B. Methods

The lymph node tissue from accessible sites (axillary, mesenteric and inguinal sites) was removed from Balb/c heart graft tolerant CBA mice at the time of sacrifice. As outlined in Chapter II, a lymph node suspension was obtained. Lymphocytes thus prepared were suspended in Leibowitz solution and injected in variable numbers ($1\text{-}10\times10^6$ lymph node cells) into irradiated (950R), cardiac allografted syngeneic recipients which received, in addition, a reconstituting inoculum of 0.1×10^6 normal bone marrow cells.

C. Results

Table 6 depicts the results to date. With tolerant donor animals derived as outlined, transfer of tolerant lymph node tissue in relatively small numbers of cells has been immuno-

Experiment	Derivation of Tolerant Animal	Tolerant Cells Transferred	Allograft survival in weeks (each line represents one animal) 2 4 6 8 10 12
1	Staput separation	2-5×10^6 LN cells	
2	Rabbit anti Balb/c antiserum treatment	2-5×10^6 LN cells	
3	Staput separation	1.8×10^6 LN cells	
4	Bone marrow + thymus	6.0×10^6 LN cells	
5	Transfer of tolerant LN	2.8×10^6 LN cells	
6	Transfer of tolerant LN	7.2×10^6 LN cells	
7	CBA anti Balb/c antiserum treatment	9.0×10^6 LN cells	
8	Reconstitution with anti-θ treated spleen	6.2×10^6 LN cells	
9	Transfer of tolerant LN	6.7×10^6 LN cells	
10	Reconstitution with anti-θ treated bone marrow	1×10^6 LN cells	
11	Transfer of tolerant LN	5.6×10^6 LN cells	
12	Transfer of tolerant LN	6.4×10^6 LN cells	
13	Transfer of tolerant LN	5.0×10^6 spleen cells	
14	Transfer of tolerant LN	1.0×10^6 spleen cells	

●Experiment terminated; ☆Graft rejected; ▶Graft activity present to date.

TABLE 6 - Transfer of tolerance with tolerant lymphocytes. Irradiated allografted mice repopulated with bone marrow + tolerant lymph node (LN) or spleen cells. Experiments are tabled in chronological order.

suppressive in every instance thus far. Eight animals reconstituted with syngeneic bone marrow and tolerant lymph node cells have become permanently graft tolerant (defined as >12 week survival of the allogeneic heart). A remaining five animals continue to exhibit graft activity at the time of writing and two animals, given 1 and 5×10^6 tolerant spleen cells with syngeneic bone marrow continue to show graft survival six weeks post-irradiation. In only one instance has functional allograft activity stopped and this occurred nine weeks post-irradiation. Since, with bone marrow reconstitution alone mean rejection occurs within 4-5 weeks post-grafting, transfer of tolerant lymph node cells confers a definite and highly significant immunosuppressive effect. Control experiments (11 animals) in which lymph node cells in small numbers were derived (5×10^6 cells) from normal animals have demonstrated that, in fashion similar to whole bone marrow reconstitution alone, rejection of the cardiac allograft occurs within five weeks post grafting.

D. Discussion

Transfer of small numbers of tolerant lymph node cells, in the system described, permits prolonged and, in most cases, permanent (>12 weeks post-irradiation) allograft survival. The mechanism whereby this effect is achieved remains obscure. The transferred lymph node cells may produce a serum blocking factor which prevents immune destruction of the graft, at least in the initial post-transplant period. Alternately, the suggestion of McCullagh (1970a) must be considered, i.e. tolerant lymphocytes can functionally repress normal (immunocompetent) lymphocytes, such as would occur in the regenerating

lymphoid system of the irradiated reconstituted mouse. This repression could, conceivably, be effected by the production of circulating humoral factor (blocking factor mediating central enhancement?) or might require direct interaction between repressor (tolerant) and normal cells.

While the bulk of evidence would favor humoral factor produced by tolerant lymphoid tissue as the most likely mechanism for tolerance transfer, there are other data which suggest that cell populations can themselves suppress or antagonize the response of another cell type. A tolerant cell in this event, could, for instance "turn off" a specific immunocompetent cell. Gershon, Cohen *et al* (1972) have found, in this regard, that histocompatible thymocyte populations can interact in a negative fashion, suppressing antigen induced immune reactivity. Allison (1971), Jacobsen, Herzenberg *et al* (1972) have similarly attributed other types of immune suppression to the direct influence of thymus derived lymphocytes. The latter authors, for example, showed that chronic allotypic suppression in mice was an active process: repopulation of such mice with nonsuppressed syngeneic spleen cells did not result in the expression of the pertinent allele. Alternately, when spleen cells of suppressed mice were transferred into normal irradiated hosts there was a short term period of production of the pertinent allele, indicating the existence of progenitor cells. Synthesis of the suppressed allotype terminated within several weeks. Evidently the spleen cells of suppressed animals contained not only cells capable of expressing the allotype but, in addition, a population of cells capable of preventing progenitor cells from

expressing their potential. Whether the suppression was exerted by direct cell to cell interaction or due to a humoral factor was unclear.

Phillips and Wegmann (1973) have produced more definite evidence for direct cell to cell interaction as a means of suppressing immune reactivity. Spleen cells from tetraparental mice did not respond to parental cells *in vitro* and, in addition, were capable of preventing immunocompetent parental spleen cells from responding either to the opposite parent or to the tetraparental cells themselves in mixed lymphocyte culture. This agreed with the earlier observations of Ceppellini (1971) that maternal cells could suppress the proliferation of paternal lymphocytes in response to newborn cells in mixed lymphocyte culture.

A number of experiments in rat chimeras do not provide evidence for cell to cell suppression as a mechanism of tolerance. For example, Wilson and Nowell (1970) found that the addition of Balb/c tolerant thymus cells did not diminish the *in vitro* sensitization of spleen cells to Balb/c alloantigens. As the previous discussion suggested, perhaps an alloantigenic stimulus to the tolerant animal prior to thymic cell harvest, could induce demonstrable suppressor cell activity *in vitro*. Search for a serum blocking factor in the sera of tolerant animals is the subject of the following chapter.

CHAPTER X

THE HELLSTRÖM ASSAY AND SERA FROM TOLERANT MICE

A. Introduction

Chapter I dealt in some detail with the work of Karl and Ingegard Hellström and associates. Hellström and Hellström (1970) published evidence that lymphocytes of tumor bearing patients are cytotoxic towards their tumor cells, but under certain circumstances, a serum factor may annul their action. This work has since been extended and now contends that the prolonged or permanent survival of an allograft or tumor is due to the specific immunosuppressive effect of a serum blocking factor, probably composed of graft derived antigen and antibody, preventing potential lymphocyte attack.

B. Materials and Methods

The details of the Hellström assay have been documented in Chapter II. Briefly, target mouse fibroblasts adhere to the bottoms of microtest wells and can be subjected to the specific cytotoxic activity of lymphocytes sensitized against target cell alloantigens. Hellström, Hellström *et al* (1971) have demonstrated that addition of serum containing blocking factor to the fibroblasts, for as little as 30 minutes, will subsequently prevent killer cell activity and fibroblasts survive incubation.

C. Results and Discussion

Sera from normal CBA mice and CBA mice tolerant to a Balb/c heart graft were exposed to Balb/c fibroblast monolayers. The fibroblasts were subsequently subjected to immune attack by CBA spleen cells which had been sensitized *in vitro*, by 96 hour incubation with Balb/c mastocytoma cells. In an initial experiment 0.1 ml of serum (diluted 1/5-1/7) was plated onto the fibroblasts (Table 7). In the presence of both normal serum and tolerant serum from five animals at this concentration, the sensitized CBA spleen cells effected significant fibroblast killing, compared with control fibroblasts exposed to cultured Balb/c spleen cells. In other words, the serum from tolerant animals did not block the cytotoxic activity of sensitized CBA spleen cells.

In a subsequent series (Table 8) tolerant sera from a further five animals were added (0.1 ml of a 1/8 dilution) to fibroblast monolayers 30 minutes following the addition of 1×10^5 sensitized spleen cells or control Balb/c spleen cells. The sera from both normal and tolerant animals in this experiment were not subsequently decanted, but incubated with lymphocytes and fibroblasts for 17 hours. Again specific killing of the target fibroblast upon presentation to a specific lymphocyte was not prevented by the presence of dilute tolerant serum.

These results suggest that blocking factors are not present in the serum of tolerant animals in the cardiac allografted mouse model. There are, however, other considerations. The sera used in these experiments was dilute and more concentrated sera might reflect blocking

Cells Added*	CBA Serum Added†	Adherent Fibroblasts (8 wells)								Mean Fibroblast Count (±S.D.)	Surviving Fibroblasts (%)
CBA-K	N	59	9	103	67	66	45	47	46	55±27	61
Balb/c	N	124	79	103	95	86	93	68	74	90±18	
CBA-K	T#1	33	30	56	39	25	50	23	-	37±12	44
Balb/c	T#1	79	84	79	50	100	126	69	-	84±24	
CBA-K	T#2	54	42	37	36	78	39	29	-	45±16	61
Balb/c	T#2	75	43	98	100	51	81	69	-	74±22	
CBA-K	T#3	18	30	40	36	43	43	44	52	38±10	49
Balb/c	T#3	56	87	76	120	68	74	74	61	77±20	
CBA-K	T#4	37	28	56	46	32	45	53	43	43±10	44
Balb/c	T#4	89	129	101	58	82	103	115	93	96±21	
CBA-K	T#5	17	53	35	69	47	46	46	-	45±16	39
Balb/c	T#5	142	196	67	83	78	-	-	-	113±55	

*CBA-K = CBA lymphocytes sensitized *in vitro* to Balb/c mastocytoma cells
Balb/c = Balb/c lymphocytes derived from tissue culture

†N = Normal CBA serum
T = Tolerant CBA serum

TABLE 7 - Hellström Assay: preincubation with tolerant sera. 0.1 ml serum diluted 1/5 - 1/7 preincubated with Balb/c fibroblasts for 30 min and then discarded; subsequent incubation period 17 hours. The killer activity of CBA-K cells on Balb/c fibroblasts was significant (p=0.01-0.001 Student's t test). The killer activity of CBA-K cells on Balb/c fibroblasts in the presence of normal and tolerant serum was not significantly different in any case (p>0.1 Student's t test).

Cells Added*	CBA Serum Added†	Adherent Fibroblasts (7 wells)							Mean Fibroblast Count (±S.D.)	Surviving Fibroblasts (%)
CBA-K	N	12	34	49	66	46	50	44	43±17	78
Balb/c		28	44	54	106	61	49	40	55±25	
CBA-K	T#1	22	20	28	26	30	52	15	28±12	57
Balb/c		28	59	48	53	39	67	51	39±13	
CBA-K	T#2	15	26	24	32	55	39	34	32±13	58
Balb/c		50	49	42	72	47	69	58	55±11	
CBA-K	T#3	57	43	46	59	47	53	24	45±12	78
Balb/c		52	57	74	51	63	43	59	57±10	
CBA-K	T#4	40	52	44	37	27	21	35	37±10	64
Balb/c		61	48	55	63	87	57	35	58±16	
CBA-K	T#5	40	35	74	48	24	37	20	40±18	68
Balb/c		87	29	57	59	47	87	46	59±22	

*CBA-K = CBA lymphocytes sensitized *in vitro* to Balb/c mastocytoma cells
Balb/c = Balb/c lymphocytes derived from tissue culture

†N = Normal CBA serum
T = Tolerant CBA serum

TABLE 8 - Hellström Assay: incubation with tolerant sera. 0.1ml serum diluted 1/8 was added 30 minutes after addition of lymphocytes; subsequent incubation period 17 hours. The killer activity of CBA-K cells on Balb/c fibroblasts was significant (p=0.01-0.001 Student's t test). The killer activity of CBA-K cells on Balb/c fibroblasts in the presence of normal and tolerant serum was not significantly different in any case (p>0.1 Student's t test).

activity. Alternately, if one considers that blocking factors act centrally to prevent expression of killer cell activity, the amount of blocking factor detectable in serum may be greatest at the time of expected rejection. Once central immunosuppression, or even peripheral enhancement phenomena had been effected, there would be very little need for excessive amounts of this factor in serum. Further, the presence of blocking factor may be antigen dependent as well as time dependent. The relatively small cardiac allograft (which does not elicit spleen cell sensitization detectable *in vitro*) may require only small amounts of humoral factor to prevent cell mediated immunity. Perhaps augmenting the antigen load by a repeat cardiac allograft prior to serum assay would elevate blocking factor activity.

The situation may even be akin to that of naturally occurring tolerance to self antigens. The irradiated animal repopulated with stem cells bears similarity to models of ontogenic development. Burnet (1972) has recently observed that his "forbidden clone" hypothesis has not yet been proven as the correct explanation for naturally occurring self tolerance. Micklem (1971), Cohen, Globerson *et al* (1972), Cohen and Wekerle (1972), Pierce (1972), and Yung, Diener *et al* (1973) have shown that "forbidden clones" may indeed exist in normal animals. Blocking factors in this regard have not yet been demonstrated; they may not occur in nonchimeric animals tolerant to naturally occurring "self" antigens.

CHAPTER XI

TOLERANCE AND TRANSPLANTATION OF THE FETAL HEART: A SUMMARY

This concluding chapter will briefly (1) summarize the work presented in the thesis, (2) suggest further relevant studies, and (3) attempt to reconcile the observations of this thesis within current hypothetical considerations.

A. Summary of Thesis Content

Allografts of fetal cardiac tissue into the subcutaneous ear tissue of syngeneic or allogeneic mice provide a convenient and precise model† for studying transplantation immunity and the induction of tolerance *in vivo*. Functional graft activity can be followed precisely by specialized electrocardiography and visual observation. The model is consistent with basic immunobiologic concepts. Syngeneic grafts are accepted permanently while allogeneic grafts are rejected within a specific time interval and exhibit characteristic histopathology. Repeat allografts, moreover, elicit an anamnestic response with accelerated rejection.

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†Appendix III documents another use of the graft model. Balb/c hearts were deep frozen (-196°C) and subsequently rewarmed by microwave thawing (Mr. R. Rajotte). They were subsequently grafted into syngeneic mice and monitored by the author. Frozen hearts were tolerant to deep hypothermia and microwave thawing and subsequently established functional graft activity.

In vivo function of the allografted heart is a most precise indication of transplantation immunity. The fetal allograft heart does elicit the production of humoral cytotoxic antibody but this does not occur in accord with rejection. There is little evidence for spleen cell mediated killer activity *in vitro* following single cardiac allografts.

Tolerance can be produced *in vivo* across a major histocompatibility barrier. This tolerance is unique in that it is induced in the adult mouse, does not involve F_1 to parent grafting or establishment of lymphoid chimerism. The model for tolerance induction involves the use of an adult CBA mouse subjected to lethal irradiation and given a Balb/c cardiac allograft. If such mice are repopulated with syngeneic whole bone marrow or spleen, allograft rejection occurs within 4-5 weeks. Several methods can produce allografts which survive beyond 12 weeks and mice bearing such allografts are considered tolerant. The stem cell rich fractions of spleen used to reconstitute irradiated allografted mice will permit small numbers of cardiac allografts to survive, even in the presence of whole bone marrow. Spleen cells subjected to velocity sedimentation separation may be more amenable to separation of stem and immunocompetent cells or, alternately, the spleen cell fraction rich in stem cells may contain a subpopulation of spleen cells with immunosuppressive activity. Tolerance can similarly be produced by treating animals post-irradiation with short term injections of homologous antisera and the IgG fractions of these sera.

Animals which reject an allograft heart following irradiation, reconstitution etc. quickly destroy a repeat Balb/c graft and are thus

immunocompetent. Mice tolerant to a Balb/c fetal heart are similarly immunocompetent: spleen cells from such animals react *in vitro* with the H-2 alloantigens of the graft and become sensitized killer cells. Despite demonstrable alloantigen reactivity *in vitro*, lymph node cells taken in small numbers from Balb/c tolerant animals specifically transfer tolerance upon injection into secondary recipients. The mechanism whereby this transfer is effected is unclear. The most recent and abundant literature would favor the production of humoral blocking factor by transferred lymphocytes. Certain other evidence suggests that tolerance induction and specific immunosuppression *in vivo* may be active processes and involve direct contact of tolerant (repressor, suppressor?) and immunocompetent cells. Apparently these mechanisms are not mutually exclusive; both may operate and their identification may depend on sensitive assay systems. In experiments performed thus far with sera from tolerant animals, blocking factor has not been evident since dilute sera did not prevent the cytotoxic activity of CBA-killer cells on fibroblast monolayers of allograft H-2 identity.

B. Future Approaches

The work described in this thesis has established several points of key interest. Allograft tolerance in the adult mouse can be obtained without lymphoid chimerism. The tolerant situation persists in the presence of alloantigen responsive cells and, further, tolerance can be transferred by tolerant lymph node cells. The nature of research is such that once a point is made, the unknown becomes more obvious, questions abound and suggest future work:

1. Humoral Aspects

   a. Are allograft directed cytotoxic and hemagglutinating antibodies apparent in the sera of tolerant animals?

   b. Is blocking factor evident in tolerant animals using

      (i) more concentrated sera

      (ii) prior alloantigen stimulation, e.g. an additional allograft some days prior to serum assay

      (iii) heart cell fibroblast monolayers as a target cell.

   c. Is blocking factor present in irradiated allografted mice given tolerant lymph node cells (clonal expansion of relevant cells)?

   d. Can the sera of tolerant mice prolong graft survival *in vivo*, such as administration of homologous antiserum to the irradiated, allografted, marrow reconstituted mouse?

2. Cellular Aspects

   a. Do lymph node cells from tolerant animals suppress mixed lymphocyte cultures (or other short term intercellular interactions) between recipient and graft strain lymphoid cells?

   b. Do lymph node cells cultured *in vitro* lose their capacity to transfer tolerance?

   c. Do lymph node cells cultured *in vitro* in the presence of alloantigen retain their ability to transfer tolerance?

   d. Is the suppressive activity of tolerant lymph node cells due to

the presence of T cells? Does anti-θ treatment remove the capacity of lymph node cells to transfer tolerance?

e. Does alloantigen recognition *in vitro* eventually wane in allograft tolerant mice?

f. What happens to tolerant lymph node cells in the irradiated mouse in terms of number and geography (T6T6 marker studies)?

g. Can allograft tolerance be broken by injecting syngeneic or allogeneic lymphocytes into tolerant mice?

3. Graft Studies

a. Would allografts which cross react (share a number of alleles) with long surviving allografts enjoy prolonged survival?

b. Does heart allograft tolerance confer tolerance to H-2 identical skin allografts?

c. Does removal of a graft from a tolerant mouse produce normal reactivity to a second allograft placed some time thereafter?

d. Do thymic grafts from tolerant mice, placed in previously thymectomized recipients promote tolerance induction?

4. General

a. Is neonatally induced tolerance characterized by alloantigen recognition *in vitro* and can lymph node cells from animals

rendered thus tolerant transfer tolerance?

b. Is adult induced irradiation chimerism associated with graft tolerance only as long as the state of chimerism persists (T6T6 marker studies) and are blocking factors more demonstrable where lymphoid chimerism exists?

C. Hypothetical Considerations

Examination of the work described in this thesis must attempt to resolve certain data.

1. Stem cell enriched fractions of spleen, but not bone marrow, will permit prolonged and even permanent heart allograft survival when used to reconstitute irradiated allografted mice.
2. Irradiated, allografted animals reconstituted with whole bone marrow reject their allografts within 4-5 weeks. Permanent heart allograft survival can be achieved with short term discontinuous administration of homologous or heterologous antisera, or the IgG component thereof.
3. Lymphocytes from tolerant animals, exposed to alloantigens *in vitro*, respond to them and become sensitized killer cells.
4. Lymph node cells from tolerant animals will transfer tolerance to secondary graft recipients.

The following hypotheses may be considered:

1. Immunocompetent cell precursors are present in both bone marrow and spleen and these are present throughout the velocity sedimentation spectrum of lymphocytes. Fraction-

ation procedures can only enrich the relative numbers of stem cells per fraction; stem cells alone cannot be isolated. The large cell fraction of spleen (sedimenting at a rate >4.5 mm/hr) contains many stem cells and immunocompetent precursor cells. In addition, however, it contains a subpopulation of thymus derived cells, not found in bone marrow, which have specific immunosuppressive activity and may be called suppressor T cells. It is the selective operation of this subpopulation of cells which can prevent emergent immunocompetent cells in both spleen and bone marrow from effecting graft rejection.

2. Antiserum treatment of irradiated, allografted animals reconstituted with whole bone marrow (containing immunocompetent cells) permits:
   a. Graft survival due to enhancement type phenomena (peripheral enhancement; antibody mediated suppression of the immune response).
   b. Graft survival due to effective removal of the immunocompetent cells that mediate graft rejection.
   c. Accumulation of that subpopulation of thymus derived cells with suppressive activity (suppressor T cells).
   d. Accumulation of humoral factors (blocking factors).

Once the short term antiserum treatment is finished, the combination of suppressor T cells and blocking factor may be sufficient to overpower immunocompetent cells responsive to graft alloantigens.

The relationship between suppressor T cells and blocking factor production is unclear. Suppressor T cells may bear a specific immunoglobulin type of receptor with avidity for circulating graft alloantigen. The T cell bearing antigen may be directly immunosuppressive by presenting antigen to relevant immunocompetent cells in a manner sufficient to elicit the Diener-Feldmann phenomenon with subsequent unresponsiveness. Suppressor T cells may provide the information to relevant B cells for specific antibody formation. This antibody, coupled with free alloantigen (perhaps T cell derived) constitutes humoral blocking factor.

3. Spleen cells from tolerant mice respond to alloantigens *in vitro* since they are effectively removed from the immunosuppressive mechanisms operative *in vivo*. Blocking factor is removed by the conditions of cell culture and the excess of alloantigen under culture conditions overrides the activity of suppressor T cells (most likely peripatetic *in vivo*) and directly stimulates immunocompetent cells.
4. The lymph node cells of tolerant mice contain suppressor T cells. Their transfer into an adult irradiated animal given small numbers of bone marrow cells provides ample time and room for their multiplication in the presence of alloantigen. The numbers of such cells are sufficient to override the small inoculum of immunocompetent cells in the bone marrow and display their suppressive activity. There are bone marrow cells present in the inoculum with which they can interact to produce humoral blocking antibody.

The summation of these events produces specific tolerance *in vivo*, and the allograft is not rejected despite demonstrable immunocompetent cells. One may postulate further, that given sufficient time the initially stimulated clones of immunocompetent cells will fall susceptible to circulating complexes of antigen and antibody and will be removed from the responsive cell pool via receptor interlinkage. The above hypothesis stresses the delicate balance between tolerance and immunity in terms of antigen, antibody and immunocompetent cells. The experimental tests for its validation are entirely feasible. It may well prove insufficient, or incorrect, but the focus imposed by such postulates can only further dispel the mysteries of transplantation tolerance.

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APPENDIX I

CELLULAR IMMUNITY *IN VITRO*

Spleen cells from CBA mice given either (1) single or multiple Balb/c cardiac allografts or (2) single flank skin grafts (Balb/c) were assessed for killer cell activity using the ^{51}Cr release assay described in Chapter II. Results of a typical series of experiments are detailed in Table 9. *In vitro* culture (96 hours) of 20×10^6 CBA spleen cells with 5×10^4 Balb/c mastocytoma cells produced killer cells capable of significant ^{51}Cr release. Skin allografts produced much less cytotoxic activity when tested at various intervals following transplantation, and the *in vitro* effect of spleen cells harvested at various intervals following cardiac allotransplantation was minimal.

The results were confirmed by Kraft (1972) using a modification of the sheep erythrocyte inhibition technique of Hirano and Uyeki (1971). In this assay the secondary *in vitro* response of CBA spleen cells to sheep erythrocytes can be inhibited by the addition of Balb/c cells sensitized against CBA alloantigens. CBA adult mice were primed three days prior to splenectomy with 0.1 ml of a 1% solution of erythrocytes injected intravenously. Primed CBA spleen cells were then cultured *in vitro* in the presence of 0.1 ml of 1% sheep erythrocytes and sensitized Balb/c spleen cells using a modified Marbrook (1967) culture technique. The total number of cells cultured per flask was 20×10^6 and the ratio of Balb/c:CBA spleen cells was either 1:1 or 1:4. Following incubation the antisheep red blood cell response was measured by counting the number of plaques using the Cunningham (1965) modification of the

	Time of Cell Harvest	Mean Supernatant Release (%)	Number of Tests or Test Animals*
CBA spleen cells sensitized by *in vitro* incubation with Balb/c mastocytoma cells	4d incubation	49.0±14.1	4
CBA recipients of single Balb/c heart grafts	7d	2.8±1.0	3
CBA recipients of single Balb/c skin grafts	7d	4.0±4.3	3
CBA recipients of single Balb/c heart grafts	10d	5.5±4.4	4
CBA recipients of multiple Balb/c heart grafts	10d	6.8±4.6	4
CBA recipients of single Balb/c skin grafts	10d	15.7±6.7	3
CBA recipients of single Balb/c heart grafts	14d	3.5±5.0	7
CBA recipients of single Balb/c skin grafts	14d	8.1±4.1	8

*Each experiment was assayed in quadruplicate reaction mixtures.

TABLE 9 - Comparison of spleen cell sensitization achieved with heart graft transplants and other procedures, as determined by the ^{51}Cr release assay.

Jerne-Nordin technique (1963). From these figures the degree of inhibition caused by the addition of sensitized Balb/c cells was determined.

Kraft (1972) found that intravenous immunization of Balb/c mice with two injections of 20×10^6 CBA spleen cells 14 days apart sensitized the Balb/c spleen cells. Their addition to CBA spleen cultures responding to sheep erythrocytes was totally inhibitory. CBA allografts were transplanted heterotopically into Balb/c mice and the Balb/c spleen cells were harvested at intervals thereafter. Their addition to CBA spleen cultures responding to sheep erythrocytes did not significantly inhibit the *in vitro* antisheep erythrocytes response at any period following allotransplantation.

APPENDIX II

IMMUNOCOMPETENT CELL DELETION BY PHARMACOLOGIC MEANS

Bruce, Meeker *et al* (1966) found that rapidly proliferating lymphoma cells were more sensitive to certain anticancer drugs (5-fluorouracil, actinomycin D, cyclophosphamide) than normal hematopoietic colony forming cells. They postulated that this differential sensitivity was due to the fact that, normally, stem cells were in a resting stage of the generation cycle and were not as susceptible to anticancer drugs as proliferating cells. This suggested that anticancer agents might be used either *in vivo* or *in vitro* to selectively deplete immunocompetent cells, while maintaining stem cell activity. The specific depletion of stimulated immunocompetent cells after BuDR exposure noted by Zoschke and Bach (1971) supported this contention.

A number of experiments were done in order to test this hypothesis. 50×10^6 irradiated (1200R) CBA spleen cells in Leibowitz solution were injected intravenously into normal male Balb/c mice, age 6-8 weeks. At various intervals thereafter the Balb/c mice were given 1-2 mgm 5 fluorouracil (5FU) intraperitoneally. This would, in theory, remove the Balb/c cells proliferating in response to CBA alloantigens. There were three groups of controls to each experiment: one group of Balb/c mice received cells but no 5FU, a second group received 5FU alone and a third group received neither cells nor 5FU. Spleen cells were harvested from Balb/c mice at various intervals and 20×10^6 cells were injected intravenously into (CBAxBalb/c)F_1 mice (males, age 8 weeks).

The activity of the injected Balb/c spleen cells was then determined for each group of five F_1 animals by spleen wt/body wt ratios eight days following injection (Cohen, Fischbach *et al*, 1970).

As expected, stimulation of Balb/c mice with a large number of CBA cells (irradiated so they could not respond) plus subsequent *in vivo* treatment with 5FU could deplete cells active in the graft-versus-host response in F_1 mice. Figure 27 details one such experiment. The allo-antigen reactivity of spleen cells from animals which received an initial injection of the antigen plus 2 mgm 5FU at 72, 96 and 120 hours and were harvested at 120 hours was markedly less than that of control groups. This depletion of immunocompetent cells was highly repeatable and the spleen wt/body wt ratio of the test group of animals does not significantly differ from that of normal (noninjected) (CBAxBalb/c)F_1 mice. When Balb/c spleen cells from test groups were tested to determine whether they retained stem cell activity, however, this had dropped precipitously. Using the colony forming assay of Till and McCulloch (1961) an injection of 1.5×10^6 normal Balb/c spleen cells into eight irradiated syngeneic recipients (900R) invariably gave rise to 15-30 splenic colonies per mouse ten days thereafter. Spleen cells from animals which had received even a single injection of 2 mgm 5FU with harvest 24-96 hours thereafter gave rise to no colonies or the occasional single colony per mouse in irradiated recipients.†
Dropping the dose of 5FU administered tenfold was necessary to retain demonstrable stem cell activity following a single injection. These quantities of 5FU were ineffective in reducing the graft-versus-host

†groups of 8 animals were injected with 1.5×10^6 syngeneic spleen cells from both control (normal) and 5FU treated mice.

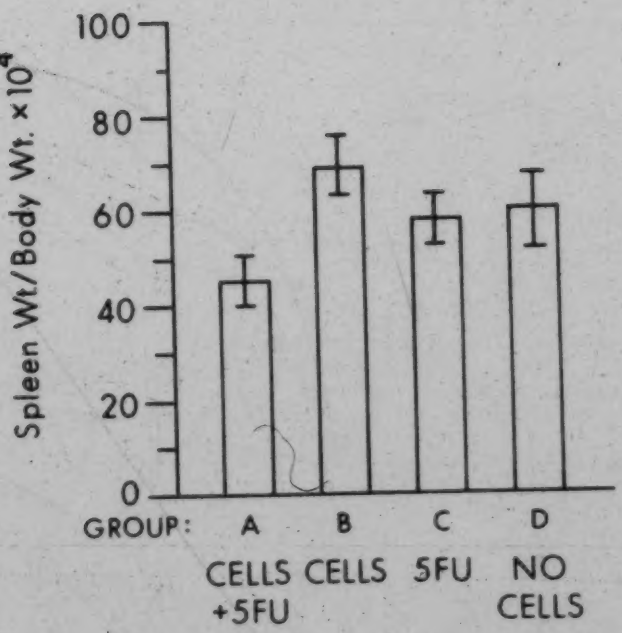

Figure 28 Immunocompetent cell depletion by pharmacologic means. Balb/c mice were given an intravenous injection of 50×10^6 CBA spleen cells (1200R) and subsequent 5 fluorouracil intraperitoneal injection.

Group A - CBA cells + 2 mgm 5 fluorouracil IP at 72, 96, 120 hours

Group B - CBA cells alone

Group C - 2 mgm 5 fluorouracil IP at 72, 96, 120 hours

Group D - no cells, no 5 fluorouracil

Spleen cells from each group harvested at 144 hours. 20×10^6 cells injected into (CBAxBalb/c)F_1 mice (5 mice per group). Spleen/body weight ratios determined 8 days thereafter.

competence of stimulated spleen cells. From these results it was reasoned that the very large amounts of 5FU required were due to the fact that relevant immunocompetent cells stimulated by antigen were variable in their time and rate of response to alloantigen (i.e. asynchronous) and required prolonged exposure to a cytotoxic agent for effective removal. This exposure was sufficient to deplete splenic stem cell activity. Because of the stem cell toxicity of 5FU treatment, this approach was considered ineffective in specific deletion of immunocompetent cells.

APPENDIX III

SURVIVAL OF ELECTRICAL ACTIVITY OF DEEP FROZEN FETAL MOUSE HEARTS AFTER MICROWAVE THAWING

Long-term preservation of organs is likely only to be achieved in the frozen state. Certain multicellular structures have already been successfully preserved after freezing. Thus, chick embryo heart primordia were successfully frozen for short periods and recovered from liquid nitrogen by Gonzales and Luyet in 1950 using ethylene glycol (EG) as a cryoprotective agent. Whittingham, Leibo *et al* (1972) froze 2-8 cell mouse embryos to -196°C and -269°C at slow cooling rates (0.3 to 2°C/min) and then thawed them slowly at rates of 4° to 25°C/min, with subsequent survival of 50-70% of 2500 such embryos developing into blastocytes on culture. When these were placed in pseudo pregnant mothers 65% became implanted as pregnancies, and of these over 40% became fetuses or went to term. Dimethylsulfoxide (DMSO), at 1M concentration, was about twice as effective as an equal concentration of glycerol. There was an optimum cooling rate and a probable need for slow as well as controlled thawing rates.

Supercooled amphibian hearts have resumed beating (Smith, 1957, 1965; Karow, 1969; Barner, 1968; Childs and Lower, 1969), but prior to the work of Offerijns and Krijnen in 1972 and Rapatz in 1970, attempts to freeze adult mammalian hearts had met with little success. A detailed review of the subject has been given by Luyet (1971).

Rapatz (1970) obtained partial resumption of activity in all parts of adult frog hearts after freezing to below -55°C, using ethylene glycol as a cryoprotective agent. Electron microscope exam-

ination of ice crystals in the tissues showed the extent to which ethylene glycol had penetrated the thick-walled ventricle compared with the thinner atria and sinus venosus, using two different freezing methods. In the first, hearts were immersed in gradually increasing concentrations of EG to 11 Molar while the tissue was cooled, step-wise, to -55°C. Some hearts were then immersed in liquid nitrogen for five minutes and then returned to 11M EG at -55°C. In the second and more successful method, which also involved step-wise decreases in temperature and increases in EG concentration, hearts were also perfused with frog Ringer's solution at various steps both in cooling to -55°C and during rewarming. Without Ringer's perfusion, sino-atrial node activity resumed in 90% but normal atrial activity resumed in only 35%; with Ringer's perfusion, spontaneous ventricular contractions also returned.

Offerijns and Krijnen (1972) added DMSO to perfusate of isolated adult rat hearts. Reversible disturbances in atrioventricular conduction occurred at normal temperatures with DMSO concentrations below 2.8M or even up to 3.5M (25% w/v) if DMSO concentration was increased slowly. With super-cooling to temperatures not below -18°C, all hearts survived in 2.1M DMSO. With freezing to -30°C, young rat hearts (10-16 days old) also recovered, but older hearts did not, possibly because of less tolerance of adult hearts to high concentrations of extracellular NaCl.

This communication reports our* experience with freezing of the fully differentiated fetal heart of the mouse, which can be reimplanted

*Work done in conjunction with Mr. R. Rajotte, Electrical Engineering Department, The University of Alberta.

in the ear of an adult syngeneic mouse and studied electrically over a long period of time. Microwave energy (25 2450 MHz) was used as one method of thawing, a method which provides uniform heating of the medium in which hearts were frozen, at a rate that can be controlled. This thawing technique may well be essential for larger organs, as it may be the only way to achieve uniformity of heating.

Seventeen to 19 day embryos were removed from the mother (Jirsch, Kraft *et al*, 1973) and their hearts placed in prechilled Cross solution (Cross and Taggart, 1949), Eagle's minimum essential medium (MEM) or in McCoy's 5a medium (the latter two also containing Hepes buffer and, in most groups, 10% fetal calf serum). Over a period of 20 minutes, increasing amounts of DMSO, DMSO with pluronic and fluorocarbon (FC47), or glycerol were then gradually added to give the final concentrations which are listed for the various groups in Table 10. Each heart, in 5 ml of solution (Table 10) was placed in a glass bottle and placed in a prechilled freezing unit (Linde BF-4-1). The freezing rate was at 0.5 to 0.7°C/min, controlled by differential thermocouples, down to -100°C. The samples were then placed in the vapour of liquid nitrogen and cooled at 5-10°C/min down to -196°C and stored for 72-216 hours at this temperature before being rewarmed.

Frozen hearts were rewarmed either by being simply placed in a water bath raising the temperature to 25°C at a rate of 100°C/min or a microwave system, similar to that recently reported for thawing tissue culture cells at different rates (Voss, Warby *et al*, 1973). In this system frozen samples are rotated horizontally on a teflon arm

Solution A -	Cross Solution, 10% DMSO
Solution B -	MEM (Eagle) with Hepes Buffer, 10% FCS, 10% DMSO
Solution C -	MEM (Eagle) with Hepes Buffer, 10% DMSO, 1% pluronic, 20% Fluorocarbon (FC47)
Solution D -	McCoy 5a Medium with Hepes, 10% FCS, 10% DMSO
Solution E -	McCoy 5a Medium with Hepes, 10% FCS, 10% Glycerol

TABLE 10 - Solutions in which hearts were frozen.

attached to a shaft which is moving up and down vertically, movement in each plane being powered by motors external to the cavity. The cavity is fed by means of a stripline radiator from a 2450 MHz magnetron, the power output of which can be varied from 0.4 to 2.0 kw. The cubic cavity is designed to resonate in a large number of modes (James, Tinga *et al*, 1968). To protect the magnetron, energy not used to warm the samples is absorbed by four beakers of water placed in the corners of the cavity. Heating rates for any frozen sample can be varied by changing the level of power input, the amount of water in the beakers and the degree of coupling from the magnetron to the cavity.

Although a high percentage of the energy is reflected back to the magnetron, the technique provides a useful method for uniform heating of samples of different shapes and sizes. Heating rates of 200°C/min were used to thaw the hearts from -196°C to 10°C±10°C. Following thawing, the embryonic hearts were reimplanted directly into the ear of the recipient mouse (Jirsch, Kraft *et al*, 1973). DMSO was not removed before reimplantation as it was felt that this concentration would not harm the tissue.

Heart survival, following reimplantation, was detected, and then monitored, by electrocardiograms (Jirsch, Kraft *et al*, 1973) on various occasions from day 5 up to day 35 as shown in Table 11.

The results of Table 11 show that Cross solution with 10% DMSO (solution A) and McCoy's solution with 10% glycerol (Solution E) were ineffective in preservation of electrical activity under the other conditions of these experiments. Similar conclusions can probably be

Mice/ Group	IF*	TS†	Thawing Method	Electrical Activity											
				Days 3-5		Days 6-10		Days 11-15		Days 16-20		Days 21-25		Days >28	
				+	%	+	%	+	%	+	%	+	%	+	%
3 5 7	B	72	MW	2/6 0/13	11	4/6 4/8	57	8/13	62	4/6 6/13 6/8	59	4/6 7/13	58	4/6 4/13	42
4 8	B	72	WB	0/4	0	4/4 4/6	80			4/4 3/6	70	4/4 3/6	70	4/4	100
9 13	D	72 216	MW			4/4	100	3/3	100	4/4	100	4/4	100		
10 14	D	72 216	WB			3/5	60	3/6	50	3/5	60	3/5	60		
1	A	72	MW	0/5	0	0/5	0			0/5	0				
2	A	72	WB	0/5	0	0/5	0			0/5	0				

*- Immersion fluid, see Table 1.
†- Time stored (hours) at -196°C
MW- Microwave warming
WB- Waterbath warming

+ Number of mice displaying electrical activity divided by Number of hearts frozen and thawed and then transplanted

% Pooled percentage survival

TABLE 11- Resumption of cardiac electrical activity after deep hypothermia. Heart grafts were transplanted into syngeneic recipients after immersion in various fluid mixtures, freezing, and either microwave or waterbath thawing.

made from the single group of hearts treated with MEM, Hepes, 10% DMSO, 1% pluronic and 20% fluorocarbon (FC47), (solution C). However, solutions B and D, both of which contain 10% FCS, 10% DMSO and Hepes buffer, in either MEM or McCoy's 5a medium have preserved electrical activity. It is with these two solutions, then, that comparison is made of the relative merits of the two methods used for thawing. The probable superiority of the microwave system over water bath immersion is shown by the earlier return of electrical activity in experimental group 3 compared to group 4, and the higher proportion that survived up to 20 days in experimental groups 7, 9 and 13 compared with 8, 10 and 14, although two hearts of group 7 ceased beating after day 28, and there was a very late recovery of one heart in group 8. Survival after freezing in solutions B or D, compared favourably with that obtained from transplanted embryonic hearts which were not frozen (Jirsch, Kraft *et al*, 1973). Some hearts have survived for several months.

Embryonic hearts at day 17-19 can withstand freezing and resume beating after thawing. The above system permits the testing of various protective agents and different freezing and thawing rates.

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